IN VITRO AND *IN VIVO* CHARACTERIZATION OF RECOMBINANT LACTOBACILLI EXPRESSING HOUSE DUST MITE ALLERGEN

LIEW LEE MEI

NATIONAL UNIVERSITY OF SINGAPORE

2009

***IN VITRO* AND *IN VIVO* CHARACTERIZATION OF RECOMBINANT LACTOBACILLI EXPRESSING HOUSE DUST MITE ALLERGEN** **LIEW LEE MEI** **2009**

IN VITRO AND *IN VIVO* CHARACTERIZATION OF RECOMBINANT LACTOBACILLI EXPRESSING HOUSE DUST MITE ALLERGEN

LIEW LEE MEI

(B.Sc., the National University of Singapore, Singapore)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PAEDIATRICS

NATIONAL UNIVERSITY OF SINGAPORE

2009

ACKNOWLEDGEMENT

First and foremost, I would like to express the depth of my gratitude to my supervisor, Professor Chua Kaw Yan, for providing invaluable knowledge, constructive ideas and constant support during my course of research study.

I would like to give a special thanks to my fellow seniors Dr Huang Chiung-Hui, Dr. Seow See Voon and Dr. Kuo I-Chun who have been providing continual guidance and ideas in this project. My gratitude continues to my fellow labmates especially Mdm Wen Hongmei, Miss Ding Ying and Mr. Andy Soh Gim Hooi for providing me technical assistance.

I also wish to thank Dr. Lars Axelsson for providing *Lactococcus lactis* subspecies *cremoris* MG1363, *Lactobacillus plantarum* strain NC8 and pSIP412 expression vector as well as his excellent technical advice in this work.

Besides, I am grateful to Dr. Lee Sanghoon, Miss Jean Xie and Miss Kelly Ong who have supported and given me time to complete my master thesis.

Last but not least, I sincerely thank my family for their love, understanding and great support throughout these years. To all my dear friends, I truly thank you for the sharing, joys and company.

TABLE OF CONTENT

SUMMARY

The prevalence of allergic asthma, allergic rhinitis and atopic dermatitis has been increasing worldwide in recent decades. The dust mite *Blomia tropicalis* is one of the main triggering factors for allergic diseases. This mite species is affecting approximately one billion people in the tropics, subtropics and certain temperate regions. Allergen-specific immunotherapy is currently the only means to alter the underlying mechanisms that lead to cure the allergic diseases. Several preclinical studies and clinical trials have suggested a possible role of lactobacilli in the prevention and treatment of allergic diseases. Lactobacilli are generally regarded as safe for oral consumption. They possess distinct adjuvant properties and exert differential immunomodulatory effects on dendritic cells (DCs). The use of lactobacilli as live vectors for oral delivery is a desirable strategy for the development of oral vaccine for allergy.

The aim of this study is to evaluate the immunomodulatory effects of *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG on DCs *in vitro* and in murine allergy models *in vivo*. The major allergen from *Blomia tropicalis*, Blo t 5, was expressed in both *Lactobacillus* strains intracellularly using the pSIP412 expression vector. Both recombinant *Lactobacillus plantarum* NC8 (rLp) and recombinant *Lactobacillus rhamnosus* GG (rLGG) could induce the maturation of bone marrow-derived dendritic cells (BMDCs) as measured by the upregulation of surface markers

and cytokine production. Furthermore, recombinant lactobacilli-pulsed BMDCs effectively activated a Blo t 5-specific T cell line. However, both recombinant lactobacilli exhibited differential modulatory effects on murine DCs as reflected by their differential cytokine production profiles.

The *in vivo* evaluation focused on the immunogenicity of recombinant lactobacilli and their protective effects against allergen-specific Th2 immune responses. Both recombinant lactobacilli-fed naive mice could elicit Blo t 5-specific B and T cell responses. In the prophylactic model, mice pre-fed with either recombinant *Lactobacillus* strain were protected against Blo t 5 sensitisation by intraperitoneal injection with Blo t 5 in alum as shown by the attenuation of Blo t 5-specific IgE, the concomitant enhancement of protective Blo t 5-specific IgG2c, and the suppression of Th2 cytokines production by Blo t 5-stimulated splenocytes and cells from mesenteric lymph nodes (MLN). In the therapeutic model, mice were adoptively transferred with Blo t 5-specific Th2 cell line and fed with recombinant lactobacilli followed by the intranasal challenge with Blo t 5. Recombinant *Lactobacillus rhamnosus* GG-fed mice showed attenuated allergic airway inflammation as manifested by the reduction of the signature cell type for allergic inflammation, eosinophils, in the bronchoalveolar lavage fluids.

In summary, recombinant lactobacilli expressing respectable levels of Blo t 5 protein have been generated and comparatively evaluated by *in vitro* and *in vivo* studies. Both recombinant lactobacilli were effective in the prevention of allergen sensitisation

despite their respective differential immunomodulatory properties *in vitro*. *Lactobacillus rhamnosus* GG was more effective than the recombinant *Lactobacillus plantarum* NC8 in the suppression of established airway inflammation. Further studies are required to address the underlying mechanisms and the clinical application in controlling the allergic diseases.

(489 words)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABSTRACTS (DERIVED FROM THIS THESIS)

Conference Abstracts:

Poster Presentations:

1. **Lee Mei Liew**, Chiung-Hui Huang, See Voon Seow, Ying Ding, Hongmei Wen, I-Chun Kuo, Kaw Yan Chua. 2007. Immunological Characterization of Recombinant *Lactobacillus plantarum* Expressing Major Mite Allergen Blo t 5. The XX World Allergy Congress 2007 (1-6 December 2007), Bangkok, Thailand.

2. **LM Liew**, CH Huang, SV Seow, Y Ding, HM Wen, KY Chua. Suppression of allergen-specific IgE production by oral administration of recombinant *Lactobacillus plantarum* in mice. The VII NHG Annual Scientific Congress 2008 (7-8 November 2008), Singapore.

3. **LM Liew**, CH Huang, SV Seow, Y Ding, HM Wen, KY Chua. Suppression of allergen-specific IgE production by oral administration of recombinant *Lactobacillus plantarum* in mice. Joint Singapore Peadistrics Congress & Asia Pacific Association of Allergology, Respirology & Immunology (APAPPARI) Meeting 2008 (3-5 October 2008), Singapore.

4. **LIEW Lee Mei**, HUANG Chuing-Hui, WEN Hongmei, KUO I-Chun, SOH Gim Hooi, CHUA Kaw Yan. Recombinant *Lactobacillus* as an oral vaccine for allergic asthma. BioMedical Asia 2009 (16-19 March 2009), Singapore.

LIST OF PUBLICATION

Publication derived from the thesis:

1. Liew LM, Huang CH, Seow SV, Ding Y, Wen HM, Kuo IC, Chua KY. Suppression of allergen-specific Th2 immune responses by oral administration of recombinant *Lactobacillus* strain in mice. (Manuscript in preparation).

Publication in the related fields:

1. Tan LK, Huang CH, Kuo IC, **Liew LM**, Chua KY. Intramuscular immunization with DNA construct containing Der p 2 and signal peptide sequences primed strong IgE production. Vaccine. 2006. 24:5762-71.

2. Huang CH, **Liew LM**, Mah KW, Kuo IC, Lee BW, Chua KY. Characterization of glutathione S-transferase from dust mite, Der p 8 and its immunoglobulin E cross-reactivity with cockroach glutathione S-transferase. Clin Exp Allergy. 2006. 36:369-76.

ABBREVIATION

3D	three-dimensional
aa	amino acid
Ag	antigen
alum	aluminum hydroxide
APC	antigen presenting cell
AU	arbitrary unit
BALF	bronchoalveolar lavage fluids
Blo t	*Blomia tropicali*s
BMDC	bone marrow-derived dendritic cell
bp	basepair
BSA	bovine serum albumin
CCL2	CC-chemokine ligand
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
cpm	count per minute
CXCL	CXC-chemokine ligand
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing non-integrin
Der f	*Dermatophagoides farinae*
Der p	*Dermatophagoides pteronyssinus*
DTT	dithiothreitol
E. coli	*Escherichia coli*
ELISA	enzyme-Linked immunosorbent assay
eos	eosinophils

Eur m	*Euroglyphus maynei*
GI	gastrointestinal
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRAS	generally regarded as safe
GST	Glutathione S-transferase
HBSS	Hanks' balanced salt solution
HDM	house dust mites
IFN	Interferon
Ig	immunoglobulin
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
IL	Interleukin
ISS-ODN	immunostimulatory oligodeoxynucleotide
kDa	kilo Daltons
LAB	lactic acid bacteria
Ll	*Lactococcus lactis* subspecies cremoris MG1363
LPS	lipopolysaccharide
LTC_4	leukotriene C_4
lym	lymphocytes
mAb	monoclonal antibody
mac	macrophages
MHC	major histocompatibility complex
MLN	mesenteric lymph node
mono	monocytes
MW	molecular weight
neu	neutrophils
ND	Non-detectable
NICE	nisin-controlled expression

NMR	Nuclear Magnetic Resonance
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pepN	aminopeptidase N
pI	isoelectric point
RBC	red blood cell
rBlo t 5	recombinant *Blomia tropicalis* group 5
rLGG	recombinant *Lactobacillus rhamnosus* GG
rLp	recombinant *Lactobacillus plantarum* NC8
rpm	rotation per minute
SCIT	subcutaneous injection
SCORAD	scoring atopic dermatitis
SEM	standard Error of Mean
SIT	allergen specific immunotherapy
SLIT	sublingual immunotherapy
SppIP	Sakacin P inducing peptide
TBS	tris buffered saline
TCR	T cell receptor
TGF	Tumor Growth Factor
Th	T helper
TNF	Tumor Necrosis Factor
Tr1	T regulatory cell 1
Treg	T regulatory
TTFC	tetanus toxin fragment C
wt	wildtype

Chapter 1

Introduction

The term “allergy”, originally coined by Clemens von Pirquet, implies deviation from the original state (von Pirquet C, 1906). However, this terminology has since been redefined and used to describe T helper 2 (Th2)-associated immune reactions to common environmental proteins, known as allergens (Kay AB, 2006). Allergen-specific Th2 cells play a central role in the development of allergic diseases such as asthma, rhinitis and atopic eczema. Over the past 25 years, the prevalence and severity of allergic diseases have reached epidemic proportions in the developed countries (Holgate ST, 1999). Allergic diseases are considered a major health problem that afflicts about 10% to 40% of the world’s populations. Billions of dollars of expenditures are being spent in the medical and health care related industry worldwide (ISAAC, 1998; Weiss KB, 2000). Allergic asthma is the most important allergic disease that is also being regarded as a common and serious respiratory disease worldwide.

1.1 House dust mite allergy-associated allergic diseases

Several reports and epidemiological studies have demonstrated that house dust mites (HDM) represent the most ubiquitous and important major indoor aeroallergens associated with allergic diseases such as allergic asthma, allergic

rhinitis and atopic eczema (Platts-Mills, 1989; Holgate ST, 1999; Ulrik CS, 2000; Lau S, 2000; Arlian LG, 2001). They play a crucial role in the pathogenesis of the allergic diseases.

Dermatophagoides pteronyssinus (Der p), *Blomia tropicalis* (Blo t), *Dermatophagoides farinae* (Der f) and *Euroglyphus maynei* (Eur m) are the most prevalent HDM species found in the world. The distribution of these domestic mite species and allergens vary geographically (Arlian LG, 2002). Humidity is the key factor for the survival of mite and their prevalence. Among them, Der p allergens are the main triggering factors for allergic diseases worldwide (Arlian LG, 1992; Thomas WR, 2002), whereas Blo t allergens are the main allergic triggering factors in the tropical and subtropical countries (Fernández-Caldas E, 1990; Puerta Llerena L, 1991; Chew FT, 1999, Kuo IC, 1999; Sanchez-Borges M, 2003; Puccio FA, 2004; Sun BQ, 2004; Yu MK, 2008). The co-existence of both Der p and Blo t allergens as well as the dual-sensitisation of atopic individual to both allergens are commonly found in the tropical and subtropical countries (Zhang L, 1997; Chew FT, 1999). Strikingly, the sensitisation of allergic patients to Blo t allergens is relatively common in Singapore and other tropical countries as compared to Der p allergens (Chew FT, 1999; Mariana A, 2000; Yeoh SM, 2003; Yi FC, 2004; Chua KY, 2007). Based on the immunochemical and cross-reactivity studies, Blo t allergens have been shown to have a relatively low to moderate cross-reactivity with Der p allergens (Chew FT, 1999). Therefore, Blo

t allergens were suggested to be included in the routine diagnostic testing for the evaluation of allergic diseases as well as the development of new preventive and therapeutic strategies in the tropical and subtropical countries (Chew FT, 1999; Puccio FA, 2004).

Approximately 1 billion people worldwide were reported to be sensitised to *Blomia tropicalis* according to the World Allergy Organization Congress, which was held in December 2007 in Bangkok. *Blomia tropicalis* is the most prevalent house dust mite species which is responsible for the provocation of allergy in tropical, subtropical and certain temperate regions with long and humid summer worldwide. In Singapore, *Blomia tropicalis* mite allergens trigger about 60% to 70% of allergic diseases such as asthma, allergic rhinitis and eczema, particularly in young allergic children. *Blomia tropicalis* group 5 allergen (Blo t 5) has been identified as the predominant allergen of *Blomia tropicalis* which sensitised up to 90% of the mite allergic patients (Arruda LK, 1995; Kuo IC, 2003; Manolio TA, 2003; Yeoh SM, 2003). Thus, there is a great demand in the development of vaccine for the management of Blo t 5-associated allergic diseases in these geographical regions. In view of the clinical importance of Blo t 5 allergen, it has been targeted as a candidate for vaccine development for the prevention and treatment of HDM-related allergic diseases.

1.1.1 *Blomia tropicalis* mite allergens

Blomia tropicalis mite was first described by van Bronswijk in 1974. This globular-shaped storage mite (Figure 1.1) is the most abundant non-pyroglyphid mite in the house dust of the tropical and subtropical countries where both the mean temperature (28ºC) and mean relative humidity (85%) are high. Its scientific classification is shown as below:

Phylum	*Arthropoda*
Class	*Arachnida*
Order	*Astigmata*
Family	*Glycyphagida*
Genus	*Blomia*
Species	*tropicalis*

Mite allergens are biochemically active molecules present in mite bodies, secreta and excreta (Tovey ER, 1981; Arlian LG, 1987). Allergenicity studies of *Blomia tropicalis* have revealed the existence of more than 20 Blo t allergens. To date, seven Blo t allergens were published with known complementary deoxyribonucleic acid (cDNA) sequences, whereas another three Blo t allergens were unpublished but submitted to the International Union of Immunological Societies (Yi FC, 2006).

Figure 1.1 Frontal view of *Blomia tropicalis* enlarged 200 times.
(Adapted from Fernández-Caldas E, Lockey RF. *Blomia tropicalis*, a mite whose time has come. Allergy. 2004. 59:1161–1164)

Blo t 5 cDNA (see Appendix 1) contains a 402-basepair (bp) open reading frame that encodes for a 17-amino acid (aa) signal peptide followed by the mature protein with 117 amino acids (Arruda LK, 1995). Its calculated molecular weight (MW) and theoretical isoelectric point (pI) value are 13.89 kilo Daltons (kDa) and 5.15, respectively. The total numbers of positively (Arg + Lys) and negatively (Asp + Glu) charged residues of Blo t 5 are 18 (15.4%) and 26 (22.2%). The extinction coefficient is low due to the lack of the tryptophan residue in Blo t 5. In addition, it has one cysteine residue and one potential N-linked glycosylation site. It is first cloned and expressed in *Escherichia coli* (E. coli) as a fusion protein with gluthathione-S-transferase (GST). Despite its 43% amino acid sequence identity to Der p 5 (see Appendix 2), the immunologlobulin E (IgE) cross-reactivity between Blo t 5 and Der p 5 is low (Arruda LK, 1995; Chew FT, 1999; Kuo IC, 2003). Even though the three-dimensional (3D) structure of Blo t 5 allergen (see Appendix 2) has been resolved by Nuclear Magnetic Resonance (NMR) (Naik MT, 2008), yet the biological function of Blo t 5 remains unknown.

1.1.2 Immunological mechanisms

HDM-associated type I allergic diseases have been regarded as an IgE-mediated hypersensitivity. They are the main source of ubiquitous indoor allergens that cause IgE-mediated sensitisation among patients with allergic rhinitis, asthma and atopic skin dermatitis (Platts-Mills, 1989; Platts-Mills, 1992). The mechanism underlying the type I allergic diseases as shown in Figure 1.2 involves a complex

series of events (Hawrylowicz CM, 2005; Larché M, 2006; Galli SJ, 2008).

Upon the inhalation and exposure of allergen, antigen presenting cells (APC) uptake and present the processed allergen to Th cells in the context of major histocompatibility complex (MHC) class II molecules (Mudde GC, 1990). The naïve $CD4^+$ T cells are then differentiated into Th2 phenotype. The differentiation and clonal expansion of allergen-specific Th2 cells lead to further production of Th2 cytokines such as interleukin (IL)-4, IL-5, IL-9, IL-13 and so forth. These Th2 cytokines are critical for the development of humoral immune response, IgE class switching as well as the pathogenesis of allergic diseases (Kips JC, 2001; Fallon PG, 2002; Georas SN, 2005). IL-4 promotes the differentiation of allergen-specific Th2 cells, whereas IL-5 leads to the activation and infiltration of eosinophils (Sanderson CJ, 1992). Eosinophils are capable of secreting cytokines such as IL-4, IL-5 and IL-6. These cytokines may act as an autocrine to increase the eosinophils survival and enhance tissue inflammation. On the other hand, IL-13 has been shown to play a role in the mucus hypersecretion, vascular permeability, smooth-muscle contraction and airway hyperresponsiveness (Grünig G,, 1998; Wills-Karp M, 1998; Montaner LJ, 1999; Corry DB, 1999; Wynn TA, 2003; Wills-Karp M, 2004). In addition, IL-9 can act on many cell types in asthma, including T cells, B cells, mast cells, eosinophils, neutrophils and epithelial cells.

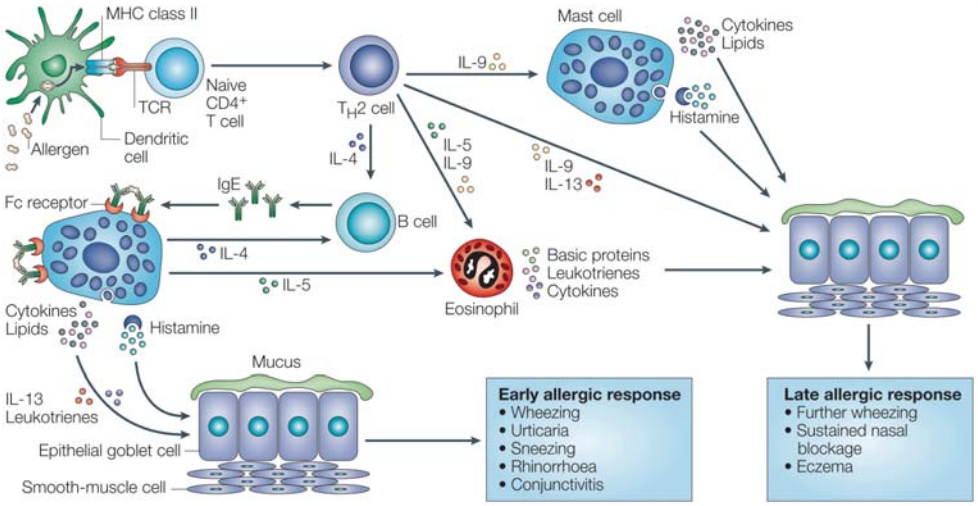


Figure 1.2 Allergic Mechanisms. In pre-disposed individuals, initial exposure(s) to allergen leads to the activation of allergen-specific T helper 2 (Th2) cells and IgE synthesis, which is known as allergic sensitisation. Subsequent exposures to allergen cause inflammatory-cell recruitment and activation and mediator release, which are responsible for early (acute) allergic responses (EARs) and late allergic responses (LARs). In the EAR, within minutes of contact with allergen, IgE-sensitised mast cells degranulate, releasing both pre-formed and newly synthesised mediators in sensitised individuals. These include histamine, leukotrienes and cytokines, which promote vascular permeability, smooth-muscle contraction and mucus production. Chemokines released by mast cells and other cell types direct recruitment of inflammatory cells that contribute to the LAR, which is characterised by an influx of eosinophils and Th2 cells. Eosinophils release an array of pro-inflammatory mediators, including leukotrienes and basic proteins (cationic proteins, eosinophil peroxidase, major basic protein and eosinophil-derived neurotoxin), and they might be an important source of interleukin-3 (IL-3), IL-5, IL-13 and granulocyte/macrophage colony-stimulating factor. Neuropeptides are also proposed to contribute to the pathophysiology of allergic symptoms. TCR, T-cell receptor.

(Adapted from Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol. 2005. 5:271-83)

One of the hallmarks of atopic diseases is the production of allergen-specific IgE. IL-4 and IL-13 favour the production of allergen-specific IgE by B cells. Other cytokines such as IL-5, IL-6 and IL-9 can enhance the IgE production, whereas interferon (IFN)-γ and IL-12 are shown to inhibit the isotype switching and production of IgE. The cross-linking of IgE-FcεRI complexes by allergen leads to the activation and degranulation of mast cells. The release of vasoactive amines such as histamine, lipid mediators such as prostaglandin D, platelet-activating factor, leukotriene C_4 (LTC_4), LTD_4, LTE_4 as well as chemokines such as CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 2 (CCL2), CCL4 and CCL5 contribute to the immediate symptoms of allergic diseases (Kay AB, 2001; Kalesnikoff J, 2001; Gould HJ, 2003). The late phase of allergic responses is characterised by the additional recruitment and activation of eosinophils and Th2 cells at the site of allergen challenge.

1.1.3 Current preventive measures and therapeutic approaches

To date, the preventive measures and therapies available for the HDM-associated allergic diseases include allergen avoidance, pharmacotherapy and allergen-specific immunotherapy (SIT).

House dust mite avoidance measures, which were proposed by Storm van Leeuwen in 1925, are widely recommended as part of a secondary and tertiary prevention strategy for allergic disease. There is some evidence to support the

preventive effect of house dust mite allergen avoidance before sensitisation occurs (Arshad SH, 2003). However, recent meta-analysis of published trials has reached a conclusion that there are no statistically significant benefits of dust mite avoidance interventions for allergic patients. It may not prevent the onset of allergic diseases in high-risk children (Arshad SH, 2004; Marks GB, 2006).

Current treatments for allergy and asthma are mainly based on pharmacotherapy. Antihistamines, corticosteroids, bronchodilators and many more are commonly used as medication for the treatment of mite allergy. These drugs effectively provide short-term relief to the patients. However, they do not suppress the rise caused by allergen exposure. Although larger doses of drugs are given, there is still no effect on the serum IgE in patients. Patients are required to take these drugs for life. It might be a burden for some families who face financial difficulties. As a result, patients' compliance and the side-effects of these drugs have become the major concerns in the treatment of allergic diseases. There is an impaired quality of life despite the adequate pharmacological treatment.

In fact, allergen-specific immunotherapy (SIT) represents the only aetiology treatment for allergic diseases, by providing allergen-specific and long-term protective immune responses. It was first introduced by Leonard Noon in the prevention of grass pollen allergy that the injection of small doses of pollen extract could induce antitoxin and ameliorate the disease (Noon L, 1911). SIT may

prevent the onset of new sensitisations in children with respiratory symptoms mono-sensitised to house dust mite (Pagno GB, 2001). This immune-modifying therapy induced immunological tolerance and the production of blocking antibodies through repeated exposure to allergens. However, this approach has potential systemic side effects and life-threatening anaphylactic responses. It may potentially induce new IgE reactivity to other components in the extracts. Besides, the long duration of treatment and the route of administration limit the patients' compliance. The standardisation of allergen extracts due to the batch variation is yet another major issue.

Conventional SIT involves repeated administration of the sensitising allergen extracts by subcutaneous injection or more recently, by sublingual route. Subcutaneous injection (SCIT) involves the regular subcutaneous injection of allergen extracts or recombinant allergens using incremental regimes. Most regimes used consists of a build-up phase (weekly injections) followed by a maintenance phase (monthly injections). Although the duration of the regime is long, the tolerance can last for several years once it is induced. It has been shown to be efficacious for the treatment of type I allergy (Werfel T, 2006; Bussmann C, 2007). On the other hand, sublingual immunotherapy (SLIT) is now accepted as a valuable alternative to SCIT for the treatment of allergic diseases. Based on the ground of safety, long-lasting efficacy, compliance and ease of applicability, it has been so far favoured in human use especially in children (Rienzo VD, 2003;

Rienzo VD, 2005; Passalacqua G, 2006; Pajno GB, 2007; Passalacqua G, 2007). Despite a few promising clinical studies showing the efficacy in the treatment of HDM-associated allergic diseases, the underlying immunological mechanisms of SLIT have only begun to be investigated which includes the time of application, duration of treatment, dosages and formulation of the allergens.

Furthermore, the application of modern bioinformatics tools and elucidation of the three-dimensional structure of major allergens have led to the allergen identification and sequencing, production of recombinant allergens, identification of B-cell and T-cell epitopes, as well as the tertiary structural analysis of allergen molecules for allergen research. Site-directed mutagenesis, expression of polypeptide fragments and introduction of single amino acid approaches have been commonly used to generate hypoallergens with reduced IgE reactivity and allergenicity while retaining their structural requirements for T-cell reactivity and induction of blocking antibodies against natural allergens. These hypoallergens are safer and more effective potential candidates for allergen-specific immunotherapy. Structurally modified hypoallergens have been produced for house dust mite (Smith AM, 1996; Takai T, 1997; Takai T, 2000, Yasuhara T, 2001), pollen, venom, food, and latex allergens, with some showing promising characteristics from the preclinical studies.

Moreover, DNA vaccination with plasmid DNA encoding allergen preferentially prime allergen-specific T helper 1 (Th1) immune response and has been postulated to potentially down-regulate the Th2 responses. The protective effects of DNA vaccine against HDM allergy have been demonstrated in murine studies with reduced infiltration of inflammatory cells, allergen-specific IgE and Th2 cytokines (Hsu CH, 1996; Jarman ER, 2004; Kim N, 2006). Thus, DNA vaccination is an alternative option for the prevention and reversal of Th2-mediated allergic hypersensitivity (Chua KY, 2006).

1.2 The history and definition of probiotics

The term "probiotics" was derived from the Greek and literally translated as "for-life". In the beginning of the last century, Eli Metchnikoff, the Russian-born Nobel Prize recipient working at the Pasteur Institute first suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff E, 1907). Metchnikoff was the first to describe the therapeutic potential of lactic acid bacteria (LAB) (Metchnikoff E, 1910). He proposed that LAB could minimise, or prevent, the harmful effects of putrefactive microbes that cause gastrointestinal (GI) disease. Decreased number of "bifid" bacteria was found in the faeces of children with diarrhoea as compared to healthy children (Tissier H, 1906). Thereby, Henry Tissier hypothesised that

administration of these “bifid” bacteria to children with diarrhoea could help them in restoring a healthy gut flora. Yet, the word “probiotics” was not coined until the later past of the last century.

The definition of probiotics has evolved through the years. In 1974, it was described as “Organisms and substances which contribute to intestinal microbial balance” (Parker RB, 1974). In 1989, it was redefined as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller R, 1989). A few years later, a similar definition was also proposed that “A viable mono or mixed culture of bacteria which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora” (Havenaar R, 1992). More recently, Schrezenmeir and de Vrese redefined it as “A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host” (Schrezenmeir J, 2001). In October 2001, the most appropriate and widely used definition was finally developed by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations, as “Probiotics are live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host". This definition has the following characteristics (Sanders ME, 2003):

1. Probiotics must be alive. Although it is recognised that dead cells may mediate physiological benefits, it was suggested that a different term refer to these agents, as consumer and scientific understanding is that probiotics are alive.
2. Probiotics must deliver a measured physiological benefit, substantiated by studies conducted in the target host.
3. Probiotics need not be restricted to food applications or oral delivery. Probiotics used as pharmaceuticals or as topical agents are not excluded from this definition.
4. A definition of probiotics should not limit the mechanism of action. Therefore, survival of GI tract transit or impact on normal flora should not be required. For example, the delivery of lactase by, for example, *Streptococcus thermophilus*, to the small intestine was recognised as a probiotic activity.

The use of probiotics in the dairy food processing, preservation and fermentation has been practiced for over a century. They are commercially available at the counter or chiller cabinet at supermarkets as bio-yogurts, probiotic drinks or food supplements. LAB such as lactobacilli and bifidobacteria are the most common probiotic strains used (O'May GA, 2005; Furrie E, 2005). Both organisms are members of the commensal gut microflora in a healthy individual (Macfarlane S, 2004).

1.2.1 The genus *Lactobacillus*

We encounter the members of *Lactobacillus* everyday. Lactobacilli are ubiquitously and heterogeneously found (Hammes WP, 1995). Non-pathogenic gram positive lactobacilli are generally regarded as safe (GRAS) and can be found in human GI tract. In healthy humans, lactobacilli are normally present in the oral cavity (10^3-10^4 cfu/g), the ileum (10^3-10^7 cfu/g) and colon (10^4-10^8 cfu/g), as well as in the vagina (Molin G, 1993; Ahrné S, 1998; Merk K, 2005). They produce lactic acid during the carbohydrate fermentation. It is easier to grow and store lactobacilli than bifidobacteria. They are well adapted to colonise the GI tract as they are acid- and bile salt-tolerant.

1.2.2 Scientific classification of *Lactobacillus*

The genus *Lactobacillus* was first described by Beijerinck in 1901. The scientific classification of *Lactobacillus* is shown as below:

Kingdom	Bacteria
Division	*Firmicutes*
Class	*Bacilli*
Order	*Lactobacillales*
Family	*Lactobacillaceae*
Genus	*Lactobacillus*
Species	*Lactobacillus plantarum*, *Lactobacillus rhamnosus* etc.

1.2.3 Taxonomy of *Lactobacillus* genus

The genus was subsequently divided into groups based on their phenotypic characteristics such as their optimal growth temperatures and hexose fermentation pathways (Orla-Jensen S, 1919), and later on obligate/facultative, homo/heterofermentation potential (Kandler O, 1986). Phenotype and phylogeny-based nomenclatures have been recently proposed. With the advances in the molecular genetic tools, the diversity of this genus has been extensively studied based on the 16S rRNA (Collins MD, 1991). New *Lactobacillus* species are being continually identified and described in the Approved List of Bacterial Names (Euzéby, www.bacterio.cict.fr). The change in the taxonomy of *Lactobacillus* is continuous and on-going: 44 species and 11 subspecies were described in 1986 (Kandler O, 1986), 88 species and 15 subspecies in 2003 (Coeuret V, 2003) and 135 species and 27 subspecies in January 2007 (www.bacterio.cict.fr). Some *Lactobacillus* species have been renamed or reclassified under new genera. Yet, some previously identified lactobacilli may still be subjected to change.

1.2.4 Epidemiological studies on probiotics and allergy

Based on the current epidemiological and observational data, different patterns of the gut microbiota composition have been reported in the intestines of non-allergic and allergic children. The first indirect evidence was shown in the Swedish and Estonian children (Sepp E, 1997). The incidence of allergic diseases was higher in

Sweden than in Estonia. Lactobacilli are reported to be found more commonly in Estonian infants than Swedish infants. Further studies have demonstrated that atopic infants are found to be less often colonised with enterococci and bifidobacteria but have higher counts of clostridia and staphylococcus during their early life (Björkstén B, 1999; Bottcher MF, 2000; Björkstén B, 2001). Similar phenomenon is observed among the Finnish children (Kalliomaki M, 2001) and Japanese children with atopic dermatitis (Watanabe S, 2003). Moreover, an association between the intestinal microbiota and IgE responses has been reported. Higher count of clostridia than that of bifidobacteria was found in 5-year-old Estonian atopic infants with specific IgE antibodies (Sepp E, 2005). Recent studies have also demonstrated that infants with higher counts of E. coli are more susceptible to the development of atopic symptoms (Penders J, 2006; Penders J, 2007). On the other hand, *Lactobacillus*-containing product beneficially affects the homeostasis of the human faecal microbiota and may contribute to the health-promoting effects (Garrido D, 2005). These findings suggest the possible role of *Lactobacillus* in the primary prevention of allergic diseases in early life.

1.2.5 Clinical studies of probiotics in the management of allergic disorders

Lactobacilli have recently been advocated for the prevention and treatment of allergic diseases. Many clinical trials have been conducted in order to test the beneficial effects of probiotics in allergic diseases including atopic eczema, allergic rhinitis, allergic asthma and food allergy. Some clinical trials are still in

progress, whereas some studies have reported a failure of probiotics in the reduction of allergic symptoms or risk of allergic diseases (Helin T, 2002; Brouwer ML, 2006; Taylor AL, 2007). Prospective analysis of well-designed double-blinded placebo controlled clinical trials with recruitment of larger population is therefore necessary to examine the probiotic effects and assess its long-term outcomes. So far, none of these studies could draw any conclusion regarding the potential effect of *Lactobacillus* on the management of allergic diseases. Nevertheless, a number of clinical trials have been conducted showing the potential use of lactobacilli in the early prevention and treatment of allergic diseases.

1.2.5.1 Probiotic prevention for atopic eczema

To date, the use of probiotics in the prevention of atopic eczema is not widely studied. Kalliomaki M et al. first reported the reduced incidence of atopic eczema in *Lactobacillus rhamnosus* GG (LGG)-supplemented group (Kalliomaki M, 2001). In this study, mothers received capsules containing LGG daily for two to four weeks before the expected delivery and to infants in the first six months of life. The frequency of atopic eczema in children at aged two in the probiotic group was half to that of the placebo group. The preventive effect of LGG on eczema extended to the age of four years, with no reduction in respiratory allergy and IgE titers (Kalliomaki M, 2003). Moreover, other studies have also shown that probiotics may reduce the incidence of IgE-associated eczema during early

infancy (Abrahamsson TR, 2007; Kukkonen K, 2007).

1.2.5.2 Probiotic treatment for atopic eczema

On the other hand, atopic eczema is the most widely studied disease by probiotic treatment. The down-regulation of allergic reactions and GI inflammation was first demonstrated in infants with atopic dermatitis and cow's milk allergy (Majamaa H, 1997). The scoring atopic dermatitis (SCORAD) scores improved significantly in the LGG-supplemented group as compared to the placebo group. The second randomised controlled trials provided another clinical demonstration of specific probiotics modifying the changes related to allergic inflammation (Isolauri E, 2000). Two months after the weaning to extensively hydrolysed whey formula (eHF), a significant improvement in the SCORAD score was observed in the atopic patients treated with LGG or *Bifidobacterium lactis*. The probiotic effect was extended at the sixth month of the follow-up study.

Moreover, the efficacy of oral supplementation of viable and non-viable LGG was assessed in the infants with atopic dermatitis and allergy to cow's milk (Kirjavainen PV, 2003). A greater improvement in the SCORAD score was obtained in the viable LGG-supplemented group. As the treatment with non-viable LGG was associated with adverse GI symptoms and diarrhoea, this study was terminated early. The premature termination of this study has suggested that supplementation of infant formulas with viable but not heat-inactivated LGG is a

potential approach for the management of atopic eczema and cow's milk allergy.

In addition, older children with more severe atopic eczema were recruited in a randomised, double-blinded, placebo-controlled, crossover trial (Rosenfeldt V, 2003). The mean SCORAD scores were improved in patients receiving the combined probiotic treatment of *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 122460 for six weeks.

So far, the largest randomised, double-blinded, placebo-controlled study was conducted using 230 infants with moderate to severe eczema (Viljanen M, 2005). Disappointingly, no differences were found in the SCORAD scores between the probiotic group and placebo group. However, in a subgroup analysis, the LGG group showed a greater reduction in the SCORAD scores compared to the placebo group in IgE-sensitised infants.

1.2.5.3 Probiotic treatment for allergic rhinitis and asthma

In 1997, a study was performed to test the probiotic effect of fifteen adolescents and adults with allergic asthma (Wheeler JG, 1997). No significant difference was noted in the clinical parameters of asthmatic patients. Another clinical study in the treatment of allergic rhinitis was conducted in 33 adults and adolescents allergic to birch pollen (Helin T, 2002). The treatment commenced 2.5 months before and finished two months after the birch pollen season. However, the treatment did not

alleviate any of the allergic symptoms or use of medication in patients. On the other hand, *Lactobacillus paracasei* have been demonstrated to be effective in improving the overall quality of life for HDM-induced perennial allergic rhinitis patients (Wang MF, 2004; Peng GC, 2005). Thus, there is so far no strong evidence supporting the role of probiotics in the treatment of allergic rhinitis and asthma.

1.3 Oral delivery of vaccines

To date, there has been much attention in the development of an oral delivery vaccine. It offers a number of advantages over the parenteral vaccination. It causes no pain and less discomfort especially when repeated or routine administration is needed. It eliminates the possibility of cross-contamination and infections caused by injection. It does not require trained personnel and the oral vaccination is therefore relatively inexpensive. It is by far the easiest and most convenient means among the different routes of mucosal immunisation. Therefore, oral vaccination represents a better choice of administration as compared to the parenteral vaccination.

Antigen degradation in the GI tract is a major hurdle in the oral delivery of antigen. As a result, the oral administration of antigen is always ineffective due to the antigen degradation in the GI tract prior to the priming and induction of an

immune response (Lee VHL, 1991). Several delivery systems have been devised to circumvent such obstacles. In general, there are two common ways to achieve the efficiency of oral delivery of antigens. A variety of synthetic delivery systems including nanoparticles, liposomes, and chitosan are currently being investigated. The use of viral or bacterial vectors represents another approach to antigen delivery via oral administration. The commonly studied viral or bacterial vectors are LAB and pathogenic microorganisms such as E. coli, *Salmonella typhimurium, Listeria monocytogenes, Vibrio cholera, Mycobacterium bovis BCG*.

1.3.1 Unique features of lactobacilli as antigen-based oral delivery vehicles

Over the past decade, there has been a growing interest in the use of lactobacilli as safe oral delivery vehicles. Some of the unique factors attributed to the selection of lactobacilli functioning as oral delivery vehicles are summarised as below:

1. They are GRAS for human oral consumption and exert health-promoting effects.
2. They can survive passage through human GI tract and possess adherence and colonisation capacities along human GI tract.
3. They can be cultured easily and manipulated genetically, thus they can be engineered to express targeting antigen and possess intrinsic adjuvant properties to elicit antigen-specific immune responses.

Lactobacilli with GRAS status are widely used in the food industries. As

compared to those pathogenic bacteria, lactobacilli can be administered live and have been consumed in large amounts by humans without causing any known detrimental health problems over centuries. Therefore, the use of live lactobacilli as oral delivery vehicles may eliminate issues that could be associated with attenuated pathogenic bacteria. In addition, acid- and bile salt-resistant lactobacilli can survive passage through human GI tract. They have overcome the major hurdle in the oral delivery of antigens. The adherence and colonisation capacities are also particularly attractive features of lactobacilli but not lactococci. Adhesion to the GI tract is a key factor affecting the capacity of lactobacilli to persist within the GI tract and to act as an adjuvant for the enhancement of specific immune response to orally administered antigen (Fang H, 2000; Plant LJ, 2002). As a result, lactobacilli are capable of eliciting local and systemic immune responses, thereby exerting their health promoting effects in human. As compared to bifidobacteria, lactobacilli are easier to be cultured and manipulated. In addition, a number of expression vectors were designed and constructed for the use in lactobacilli. The advances in the molecular tools for genetic manipulation have allowed genetically engineered *Lactobacillus* strains to function as live antigen delivery vehicles for targeting antigens to mucosa tissues (Pouwels PH, 1996; Pouwels PH, 1998; Pouwels PH, 2001), thereby effectively inducing antigen-specific protective immune responses (Kruger C, 2002; Pant N, 2006). Taken altogether, these attractive features make lactobacilli better choices for the development of safe and efficacious antigen-based oral delivery vaccines.

1.3.2 Mechanisms of lactobacilli as immunomodulator

Mucosal surfaces represent a very large proportion of the surface area of the human body. The mucosa of the small intestine alone is estimated to be 300 m^2 in humans (Moog F, 1981). Dendritic cells (DCs) are particularly abundant at mucosal sites and are recruited during infections to the site of mucosal inflammation. DCs are professional antigen presenting cells (APCs) which play a key role in the antigen sampling, early bacterial recognition, regulation of T cell immune responses as well as the induction of immunological tolerance (Banchereau J, 1998; Kaliński P, 1999; Kronin V, 2000; Banchereau J, 2000; Kapsenberg ML, 2003; Kelsall BL, 2004; Iwasaki A, 2007; Coombes JL, 2008).

The induction of T cell responses requires three signals from DCs. Upon the exposure to antigen or microbial stimulation, the MHC class II and co-stimulatory molecules are upregulated on DC surface. DCs migrate to the draining lymph nodes. Followed by DC maturation, DCs present processed antigen to naïve T cells, thereby priming and inducing adaptive immune responses. The formation of MHC-Ag complexes is the first signal that determines the antigen specificity of the response. The second signal is provided by the co-stimulatory molecules expressed on DCs during the DC maturation. In the absence of the co-stimulatory molecules, Th cells become anergic which might lead to tolerance. In addition, mature DCs secrete multiple cytokines for the subsequent T cell differentiation and polarisation.

DC-derived cytokines play the most important role in the T cell polarisation towards Th1 or Th2 or T regulatory (Treg) cell development (Mazzoni A, 2004). Interleukin-12 (IL-12) family members such as IL-12, IL-23 and IL-27 have been reported as strong Th1-skewing cytokines which mediate the production of IFN-γ by Th1 cells (Hsieh CS, 1993; Heufler C, 1996; Mosmann TR, 1996; O'Garra A, 1998; Trinchieri G, 2003). DCs produce IL-12 upon interaction between CD40 on the APC and the rapidly induced CD40 ligand (CD40L, CD154) on the activated Th cell (Koch F, 1996; Cella M, 1996; Hilkens CM, 1997). Conversely, exposure of naïve T cells to IL-4, IL-5, IL-10 and IL-6 results in Th2 cell development. Notably, IL-10 is a pleiotropic cytokine that modulates the function of several adaptive immunity-related cells by promoting either Th2 immune response or the generation of Treg cells with an immunosuppressive function (Mocellin S, 2004). IL-10 and transforming growth factor-β (TGF-β) are well documented as regulatory T cell-polarising factors (Groux H, 1996; Zeller JC, 1999; Akbari O, 2001). IL-6 is mainly produced by APCs. IL-6 promotes terminal differentiation of B cells into plasma cells, induces Th2 cell polarisation (Rincón M, 1997), and enhances the intestinal IgA response (McGhee JR, 1991; Ramsay AJ, 1994; Braciak TA, 2000).

Recent findings have suggested that *Lactobacillus* strains, with their own intrinsic adjuvant properties, could differentially modulate the surface marker expression and cytokine production by DCs, with the possibility to favour Th1, Th2 or Th3

immune responses (Christensen HR, 2002; Drakes M, 2004; Hart AL, 2004; Mohamadzadeh M, 2005; Niers LE, 2007). In addition, Smith et al. has demonstrated that *Lactobacillus* strains, which bind the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), can actively prime DCs to the development of IL-10-producing Treg cells (Smits HH, 2005). This suggests that *Lactobacillus* strains, which are potent IL-12 or IL-10 inducers, can differentially modulate DCs, thereby promoting Th1 or Treg cell development to antagonise Th2 immune response.

1.3.3 Strain selection

The proper selection of *Lactobacillus* strains is crucial for the development of an oral vaccine for the management of allergic diseases. Based on the effects of their intrinsic adjuvanicity, some *Lactobacillus* strains could potentially suppress the Th2-mediated allergic diseases by the immune deviation to Th1-type immune response (Murosaki S, 1998; Repa A, 2003; Hisbergues M, 2007) or Treg activation.

1.3.3.1 *Lactobacillus plantarum*

Lactobacillus plantarum is a potent inducer of IL-12, directing the Th1 cell development (Hessle C, 1999; Repa A, 2003). *Lactobacillus plantarum* is more immunogenic than *Lactococcus lactis* (Grangette C, 2004; Cortes-Perez NG, 2007). *Lactobacillus plantarum* is shown to induce IL-10 production by DCs and

$CD4^+$ T cells, thereby enhancing the efficacy of SLIT (Van Overtvelt L, 2008). Furthermore, *Lactobacillus plantarum* has been used in the studies of birch pollen (Repa A, 2003; Daniel C, 2006) and dust mite allergy (Pochard P, 2005; Hisbergues M, 2007). Studies have shown that *Lactobacillus plantarum* is capable of inducing Th1- or Treg-type immune responses (Repa A, 2003) and suppressing the airway inflammation in mice (Daniel C, 2006). The co-administration of *Lactobacillus plantarum* and mite allergen is capable of inducing the production of IL-10 and IL-12 by monocyte-derived DCs from allergic patients (Pochard P, 2005). This suggests that *Lactobacillus plantarum* might have the capacity to switch the established Th2 response in mite allergic patients towards Th1 response. Besides, the co-administration of *Lactobacillus plantarum* and Der p 1 has been shown to prevent the development of the allergic response in mice (Hisbergues M, 2007). More recently, recombinant *Lactobacillus plantarum* expressing Der p 1 represents a promising prophylactic vaccine against mite allergy. It has been shown to induce antigen-specific immune responses and reduce allergic airway eosinophilia upon the aerosol challenge of allergen (Rigaux P, 2009).

1.3.3.2 *Lactobacillus rhamnosus* GG

Lactobacillus rhamnosus GG (LGG) is one of the best-studied probiotic bacteria. Clinical trials have shown that LGG effectively prevents early atopic disease in children at high risk (Kalliomaki M, 2001; Kalliomaki M, 2003). Treatment with LGG has led to an increase in the production of IFN-gamma in infants with cow's

milk allergy (Pohjavuori E, 2004) and the alleviation of atopic eczema/dermatitis syndrome in IgE-sensitised food allergic infants (Viljanen M, 2005). In addition, Feleszko W et al. has reported that the administration of LGG suppresses the asthmatic phenotype such as airway reactivity, antigen-specific immunoglobulin E production and pulmonary eosinophilia in newborn mice. An induction of T regulatory cells associated with increased TGF-β production is observed (Feleszko W, 2007). Several reports suggest that the bacterial cell wall components and soluble factors of LGG might contribute to its immunomodulatory ability (Miettinen M, 2000; Matsuguchi T, 2003; Pena JA, 2003). More recently, LGG-derived immunostimulatory oligodeoxynucleotide (ISS-ODN) ID35 is shown to exert anti-allergic effect by activating DC, eliciting effective Th1 responses and suppress antigen-specific IgE production *in vivo* (Iliev ID, 2008). The effect of ID35 on switching the Th bias to a non-allergic Th1 response and on the suppression of ovalbumin (OVA)-specific IgE production was comparable with the effect induced by murine prototype CpG 1826.

Taken together, *Lactobacillus plantarum* and LGG might represent potential candidates for the management of mite-associated allergic diseases.

1.4 The rationale of the study

Recent studies have demonstrated that Blo t 5 is the major indoor HDM allergen in the tropical and subtropical countries such as Singapore, Malaysia, Hong Kong, Thailand, Taiwan and many more. In view of the clinical importance of Blo t 5 in these geographical regions, its role as a part of armamentarium of therapeutic agents used for the allergen-specific immunotherapy for allergic diseases should not be neglected.

Defects in the development of regulatory T cells have been implicated in the development of allergic diseases. Strategies that restore immune regulation may potentially be exploited as clinical interventions. Probiotics is one such modality and has been used in clinical trials for the prevention and treatment of allergic diseases and inflammatory bowel disease. Hygiene hypothesis has also suggested a possible role of *Lactobacillus* in the primary prevention of allergic diseases early in life. Some epidemiological studies have revealed that the composition of the gut microbiota differs in healthy and allergic infants. Moreover, several clinical studies have showed promising data on lactobacilli effects in atopic diseases. Some preclinical studies using allergen-expressing recombinant lactobacilli have also demonstrated that it could be a promising strategy to attenuate allergic airway inflammation. Taken together, recombinant lactobacilli are potential candidates for the development of antigen-based oral vaccines in the primary prevention and management of allergic diseases.

Experimental data have indicated that *Lactobacillus plantarum* is a potent IL-12 inducer, whereas LGG is a potent IL-10 inducer. Therefore, *Lactobacillus plantarum* NC8 and LGG are chosen and genetically engineered to express Blo t 5 in this project. The hypothesis of this study are that (1) recombinant *Lactobacillus plantarum* NC8 and recombinant LGG expressing Blo t 5 could induce Blo t 5-specific immune responses which can antagonise the Blo t 5-mediated Th2 immune response; (2) *Lactobacillus plantarum* NC8 and LGG exhibit differential intrinsic adjuvanicity that may lead to differential immunomodulatory effects on differentiation of antigen-specific T subsets.

To date, LGG has not been explored and studied as a host strain expressing allergen. This study is the first to explore and compare the immunomodulatory and protective effects of recombinant *Lactobacillus plantarum* NC8 with recombinant LGG against allergic reactions.

1.5 The specific aim and experimental strategies of the study

The specific aim of this study is to evaluate the protective and immunomodulatory effects of recombinant *Lactobacillus* strains expressing Blo t 5 in prevention and treatment of allergic asthma in a mouse allergy model.

The experimental strategies used in the study are as followed:

1. Construction of plasmid pSIP412 containing the cDNA encoding for the major mite allergen Blo t 5 (designated as pSIP412-Bt5)
2. Generation of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG expressing Blo t 5 (designated as rLp and rLGG, respectively)
3. Evaluation of the expression levels of Blo t 5 in recombinant *Lactobacillus plantarum* NC8 and recombinant LGG
4. Evaluation of the effects of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG on the phenotype and cytokine production profiles of bone marrow-derived dendritic cells (BMDCs) and Flt3-derived DCs
5. *In vivo* evaluation of the immunogenicity of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG in naïve mice
6. *In vivo* evaluation of the anti-allergic immunomodulatory effects of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG in mouse allergy model.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Yeast and bacterial strains

Pichia pastoris strain KM71 [Genotype: *aox1::ARG4*, arg4 Mut^{s}] was purchased from Invitrogen (Carlsbad, CA, USA) and used for the expression of recombinant Blo t 5.

Lactococcus lactis subspecies *cremoris* MG1363 was used as intermediate host for plasmid propagation. *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG (ATCC 53103) were used as final hosts for protein expression. The characteristics of the bacterial strains used are listed in the Table 2.1. *Lactococcus lactis* subspecies *cremoris* MG1363 and *Lactobacillus plantarum* NC8 were generously provided by Dr. Lars Axelsson (Matforsk, Norwegian Food Research Institute, Osloveien, Norway).

Table 2.1 The characteristics of plasmid and bacterial strains used in this study

Plasmid or strain	Characteristics	Source	Remarks
pSIP412	Em^R, inducible P_{orfX}, *Lactococcus lactis* pSH71 replicon, pepN reporter gene	Sørvig E (1983)	Vector map (Appendix 5), DNA sequence (Appendix 6)
Lactococcus lactis subspecies cremoris MG1363 (Ll)	Host strain, plasmid-free, prophage-cured derivative of *Lactococcus Lactis* NCDO 712	Gasson MJ (1983)	
Lactobacillus plantarum NC8 (Lp)	Host strain, silage isolate, plasmid-free	Aukrust T (1992)	
Lactobacillus rhamnosus GG (LGG) (ATCC 53103)	Host strain, human isolate, plasmid-free	Silva M (1987)	

2.1.2 Yeast and bacterial culture media

Yeast nitrogen base (YNB), Bacto-yeast extract, Bacto-peptone, Bacto-tryptone, Bacto-agar, gelatin and MRS broth were purchased from Difco Laboratories (Sparks, MD, USA). M17 broth and M17 agar were purchased from Oxoid (Basingstoke, Hampshire, England). Sodium chloride, potassium phosphate monobasic, potassium phosphate dibasic, magnesium chloride, calcium chloride and methanol and were purchased from Merck (Whitehouse station, NJ, USA). Glycerol was purchased from Univar (Kirkland, WA, USA). Glycine, glucose, histidine, biotin and erythromycin were purchased from Sigma (St. Louis, MO, USA). Sucrose was purchased from 1st Base Pte Ltd (Singapore).

2.1.3 Reagents for protein purification, identification and analysis

Butyl Sepharose 4 Fast Flow and Q-Sepharose Fast Flow were purchased from Pharmacia (Uppsala, Sweden). Blo t 5-specific monoclonal antibody (mAb) 4A7 was generated in our laboratory as previously described (Yi FC, 2005). Biotinylated anti-mouse immunoglobulins and ExtrAvidin® Peroxidase conjugate were purchased from Sigma (St. Louis, MO, USA). Hybond^{TM}-C Extra nitrocellulose membrane was purchased from Amersham Biosciences (Amersham Place, Little Chalfont, Bucks, UK). Bio-safe Coomassie stain was purchased from Bio-Rad (Hercules, CA, USA). SuperSignal West Pico Chemiluminescent Substrate and Kodak BioMax film were purchased from Pierce (Rockford, IL, USA) and Eastman Kodak Company (Rochester, NY, USA), respectively.

2.1.4 Plasmid and reagents for molecular cloning

pSIP412 expression vector (Figure 2.1, Appendix 5) was a kind gift from Dr. Lars Axelsson (Matforsk, Norwegian Food Research Institute, Osloveien, Norway). *Taq* DNA polymerase was purchased from Roche (Indianapolis, IN, USA). Klenow polymerase, *Xho I*, *Nco I*, T4 DNA ligase and Wizard® Plus SV miniprep DNA purification system were purchased from Promega (Madison, WI, USA). GFX PCR DNA and gel band purification kit was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). BigDye® Terminator v3.1 Cycle Sequencing R-100 Kit was purchased from Applied Biosystems (Foster City, CA, USA). Lysozyme from chicken egg white was purchased from Sigma (St. Louis, MO, USA).

2.1.5 Mice

Female C57BL/6J mice, 4-6 weeks of age, were purchased from Centre for Animal Resources (CARE) at Lim Chu Kang and housed under conventional condition (temperature 22ºC and relative humidity 65%) in the National University of Singapore (NUS) Animal Holding Unit (AHU). Food and water were available *ad libitum*. All experiments were performed according to the Institutional Guidelines for Animal Care and Handling (IACUC).

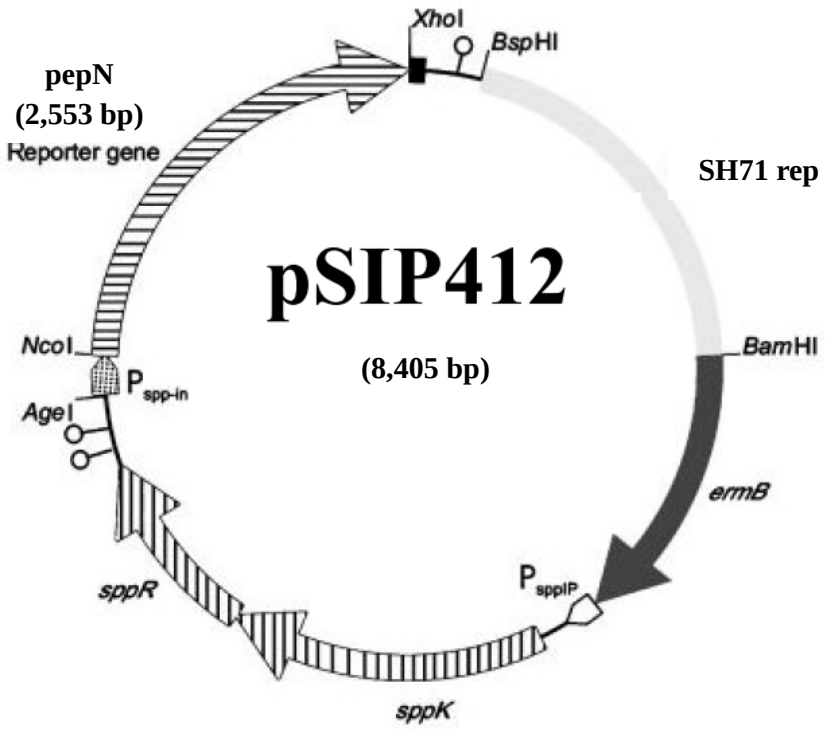


Figure 2.1 Schematic diagram of the pSIP412 expression vector. Horizontally hatched regions: reporter gene (pepN); Dotted regions: inducible promoter (P_{orfX}); Light-grey regions: replication determinants (SH71rep); Dark-grey regions: erythromycin resistance marker (*ermB*); White regions: inducible *sppIP* promoter (P_{sppIP}); Vertically hatched regions: histidine protein kinase and response regulator genes; Lollypop structures, transcriptional terminator; Black boxes: multiple cloning sites (MCS; *SphI, XbaI, XhoI, EcoR1, KpnI, SmaI, NarI, HindIII*). All restriction sites are unique.

2.1.6 Inducing peptide for protein induction

Sakacin P inducing peptide (*SppIP*, MAGNSSNFIHKIKQIFTHR, $C_{99}H_{157}N_{31}O_{26}S_1$) was synthesized by 1st Base Pte Ltd (Singapore). The purity is > 90% by HPLC.

2.1.7 Reagents for mice immunization

Amphojel® Aluminum hydroxide (alum) was purchased from Whitehall Laboratories Pty Ltd (Australia).

2.1.8 Reagents for cell culture

Hanks' balanced salt solution (HBSS) without calcium and magnesium was purchased from Sigma (St. Loius, MO, USA). RPMI 1640 medium, penicillin, streptomycin, L-glutamine and sodium pyruvate were purchased from Hyclone (Logan, Utah, USA). Fetal bovine serum was purchased from PAA Laboratories GmbH (Haidmannweg, Pasching, Austria). 2-Mercaptoethanol was purchased from Gibco (Grand Island, NY, USA). Ficoll-Paque plus was purchased from Amersham Biosciences Corp (Piscataway, NJ). OptiPrep™ density gradient media was purchased from Axis Shield Poc AS (Oslo, Norway). Mitomycin-C was purchased from Roche Diagnostic GmbH (Mannheim, Germany).

Complete RPMI 1640 medium was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml

Streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 5.5 x 10^{-2} mM 2-Mercaptoethanol. Complete RPMI 1640 medium was used for all cell culture experiments.

2.1.9 Antibodies and recombinant cytokines

Purified mouse antibodies IgE and IgG1 as well as recombinant mouse cytokines IL-2, IL-4, IL-5, IL-6 and TGF-β were purchased from BD Pharmingen (San Diego, CA, USA). Recombinant mouse cytokines IL-10, IL-12, IL-13, IFN-γ and TNF-α were purchased from R&D systems (Minneapolis, USA). Recombinant mouse GM-CSF and mouse Flt3L were purchased from PeproTec Inc. (Rocky Hill, NJ).

PE-conjugated anti-mouse CD40 (clone 1C10) was purchased from eBioscience (San Diego, CA, USA). APC-conjugated anti-mouse CD11c (clone HL3), FITC-conjugated anti-mouse I-A^b (clone AF6-120.1), FITC-conjugated anti-mouse CD80 (clone 16-10A1), PE-conjugated anti-mouse CD86 (clone GL1), purified mAbs against mouse Ig κ light chain (clone 187.1), IL-4 (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), IFN-γ (clone R4-6A2), TGF-β (A75-2.1), as well as biotin-conjugated mAbs against mouse IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), IFN-γ (clone XMG1.2), TGF-β(clone A75-3.1), were purchased from BD Pharmingen (San Diego, USA)

Purified mAbs against mouse IL-10 (clone JES052A5), IL-12p70 (clone 48110), IL-13 (clone 38213), polyclonal Abs against mouse TNF-α as well as biotin-conjugated polyclonal Abs against mouse IL-10, IL-12, IL-13 and TNF-α were purchased from R&D systems (Minneapolis, USA).

Biotin conjugated anti-mouse IgE (clone LO-ME-3) and IgG1 (clone LO-MGI-2) were purchased from Serotec (Oxford, England). ExtraAvidine Alkaline Phosphatase conjugate, Sigma Fast p-Nitrophenyl phosphatase substrate (pNPP), 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) tablets (ABTS), phosphate-citrate buffer with urea hydrogen peroxide tablet and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Mouse IgG2c ELISA Quantitation Kit was purchased from Bethyl Laboratories Inc. (Montogomery, TX, USA).

2.2 Methods

2.2.1 Purification of recombinant Blo t 5

Recombinant Blo t 5 (rBlo t 5) was produced from the *Pichia pastoris* yeast expression system (Goh LT, 2001). Briefly, the *Pichia pastoris* KM71 transformants carrying Blo t 5 cDNA were grown in 6 L of buffered glycerol-complex medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen

base with ammonium sulfate, 1% glycerol, 0.4 mg/L biotin, and 0.1 mol/L potassium phosphate, pH 6.0) until the OD_{600} = 6. Cells were harvested by centrifugation at 1000g and gently suspended in 600 mL of buffered methanol-complex medium containing 0.5% methanol. The cells were cultured for another 2 days for Blo t 5 induction. Methanol was replenished to 0.5% final concentration every 24 hours. The yeast media containing rBlo t 5 was processed by conventional fast performance liquid chromatography (Goh LT, 2001). Purified rBlo t 5 was analyzed by SDS-PAGE and confirmed by western blot. The concentration of rBlo t 5 was determined using the UV absorbance spectroscopy method.

2.2.2 Gel electrophoresis

2x SDS sample buffer (0.1 M Tris-HCl (pH 6.8), 4% SDS, 12% 2-Mercaptoethanol, 20% glycerol, 10 mM dithiothreitol (DTT) and a small amount of bromophenol blue) was added at equal volume to each protein sample. The samples were boiled for 5 mins prior to gel loading. Proteins were subsequently separated on 7.5% SDS-PAGE. Protein bands were identified by staining the gel with Bio-safe Coomassie stain solution (Bio-Rad, Hercules, CA, USA).

2.2.3 Western blotting

Proteins were separated on 7.5% SDS-PAGE and electroblotted onto Hybond^{TM}-C extra nitrocellulose membrane (Amersham Biosciences, Amersham Place, Little Chalfont, Bucks, UK). After blocking with 5% milk in tris buffered saline (TBS) containing 0.05% Tween-20 (TBS-T), the blot was washed 3 times with TBS-T and incubated with 2 ml of diluted mAb 4A7 at 4°C overnight. The blot was washed 5 times with TBS-T and incubated with biotinylated anti-mouse immunoglobulins at a dilution of 1:5000 (Sigma, St. Louis, MO, USA). The blot was washed 5 times with TBS-T and incubated with ExtrAvidin peroxidase conjugate at a dilution of 1:10000 (Sigma, St. Louis, MO, USA) at room temperature for 1 hour. The blot was washed 6 times with TBS-T and signals were developed with SuperSignal West Pico chemilumisnescent substrate (Pierce, Rockford, IL, USA) on BioMax film (Eastman Kodak Company, Rochester, NY, USA).

2.2.4 Bacterial growth conditions

Lactococcus lactis subspecies cremoris MG1363 was grown in GM17 medium. *Lactobacillus plantarum* NC8 and LGG were grown in MRS medium (Difco, Becton Dickinson, USA). *Lactococcus lactis* and *Lactobacillus* strains were grown in unshaken cultures at 30ºC. Erythromycin at a final concentration of 5 μg/ml was added as a selection antibiotic for recombinant *Lactococcus lactis* and recombinant lactobacilli. The detailed recipe of the culture media used for

Lactococcus lactis and *Lactobacillus* strains were listed in Appendix 3 and Appendix 4.

2.2.5 The construction of pSIP412-Bt5 expression vector

The construction of pSIP412-Bt5 expression vector was schematically shown in Figure 2.2. Briefly, the pSIP412 vector was digested with *Nco I* (Promega, Madison, WI, USA), treated with Klenow polymerase (Promega, Madison, WI, USA) to fill in the protruding end and further digested with *XhoI* (Promega, Madison, WI, USA). The coding sequence of mature Blo t 5 protein (see Appendix 1) was amplified from a recombinant plasmid pGEX-4T_1-Bt5 (Yi FC, 2004) by polymerase chain reaction (PCR) using *Taq* DNA polymerase (Roche, Indianapolis, IN, USA). The sequences of Blo t 5-specific sense and antisense primers (1^{st} Base Pte Ltd, Singapore) used were 5'-CAAGAGCACAAGCCAAAGAAGG-3' and 5'-CGCTCGAGTTATTGGGTTTGAATATCCTT-3', respectively. The antisense primer was designed to contain an *Xho I* restriction site (underlined). The conditions of the PCR were: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec; 72°C for 10 min. PCR product was subsequently digested with *Xho I*. The resulting plasmid and the digested PCR fragment were ligated by using T4 DNA ligase (Promega, Madison, WI, USA). The ligation mixture was incubated at 16°C overnight.

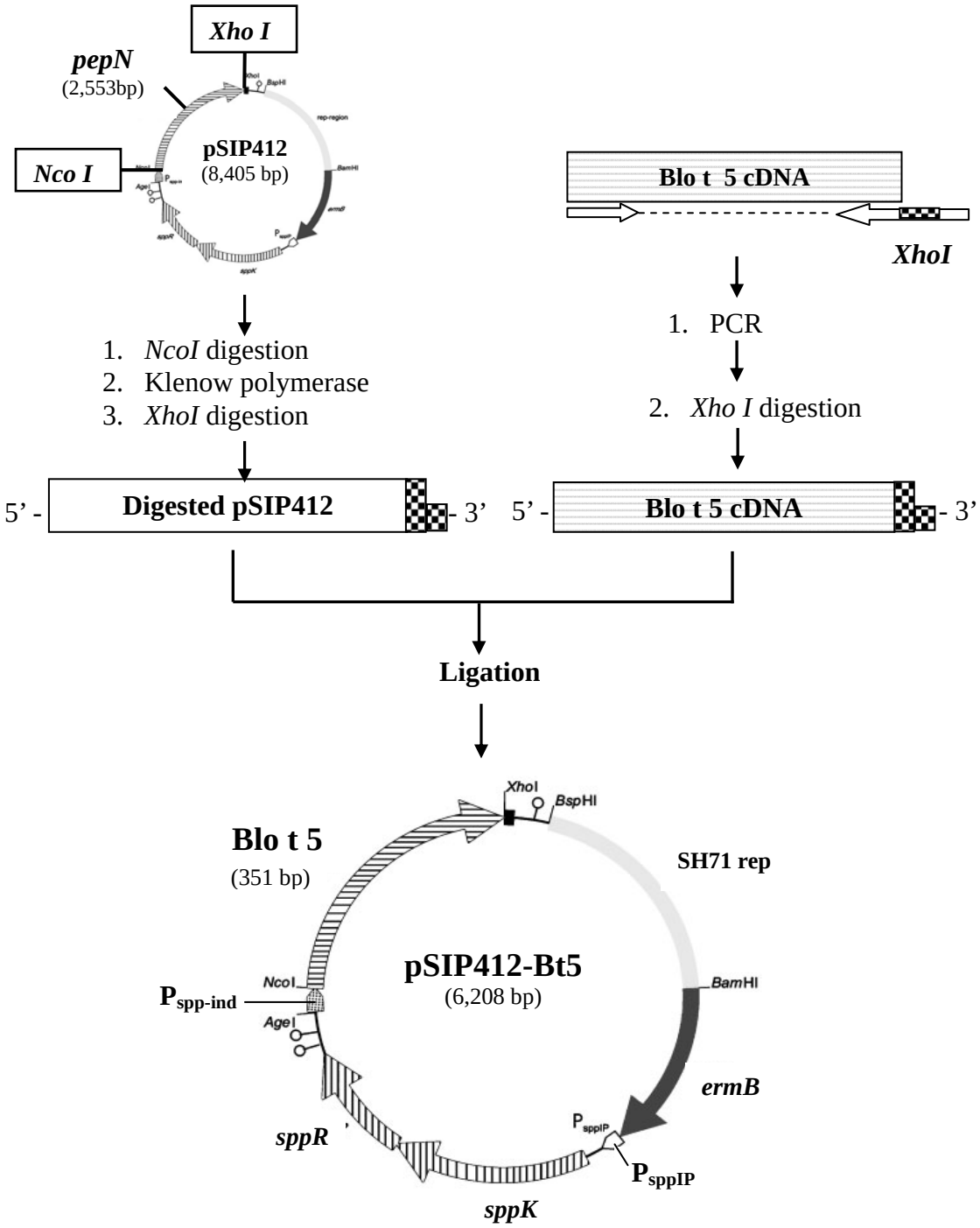


Figure 2.2 The schematic representation of the construction of pSIP412-Bt5 expression vector. The aminopeptidase N (*pepN*) reporter gene of the pSIP412 plasmid was removed by double digestion with *Ncol* and *Xhol*. The sequence encoding for the full length Blo t 5 was amplified from pGEX-4T$_1$-Bt5, digested with *XhoI* and cloned into the blunt end at Klenow polymerase-treated *NcoI* site and cohesive end at *XhoI* site of pSIP412 to generate pSIP412-Bt5.

The stepwise transformation procedures were summarized in Figure 2.3. Briefly, the ligation mixture was electrotransformed into *Lactococcus lactis* subspecies cremoris MG1363 as an intermediate host for plasmid propagation (section 2.2.6). pSIP412-Bt5 was extracted and nucleotide sequences were confirmed by DNA sequencing (section 2.2.7). Recombinant plasmid pSIP412-Bt5 was finally electrotransformed into *Lactobacillus plantarum* NC8 and LGG as final hosts for Blo t 5 expression (section 2.2.8).

2.2.6 Electrotransformation of *Lactococcus lactis*

Lactococcus lactis subspecies cremoris MG1363 was used as an intermediate host for plasmid propagation. The electroporation was conducted by using a Bio-Rad Gene Pulser Xell apparatus (Bio-Rad, Hercules, CA, USA) as described (Holo H, 1989). Briefly, the mixture of *Lactococcus lactis* competent cells and ligation mixture was transferred into an ice-cold 2 mm disposable electroporation cuvette (Bio-Rad, Hercules, CA, USA). A single pulse was delivered with the following settings: voltage, 2.0 kV; capacitance, 25 μF; resistance, 200 Ω. These settings gave a time constant of 4-5 ms. Immediately following the pulse, bacteria were resuspended in SGM17MC and incubated at 30ºC for 2 hours. Transformants were selected by plating the bacteria on SR agar plate containing 5 μg/ml of erythromycin. Transformants were visible after incubation at 30ºC for 2 to 3 days. The detailed recipe of the culture media used for *Lactococcus lactis* was listed in Appendix 3.

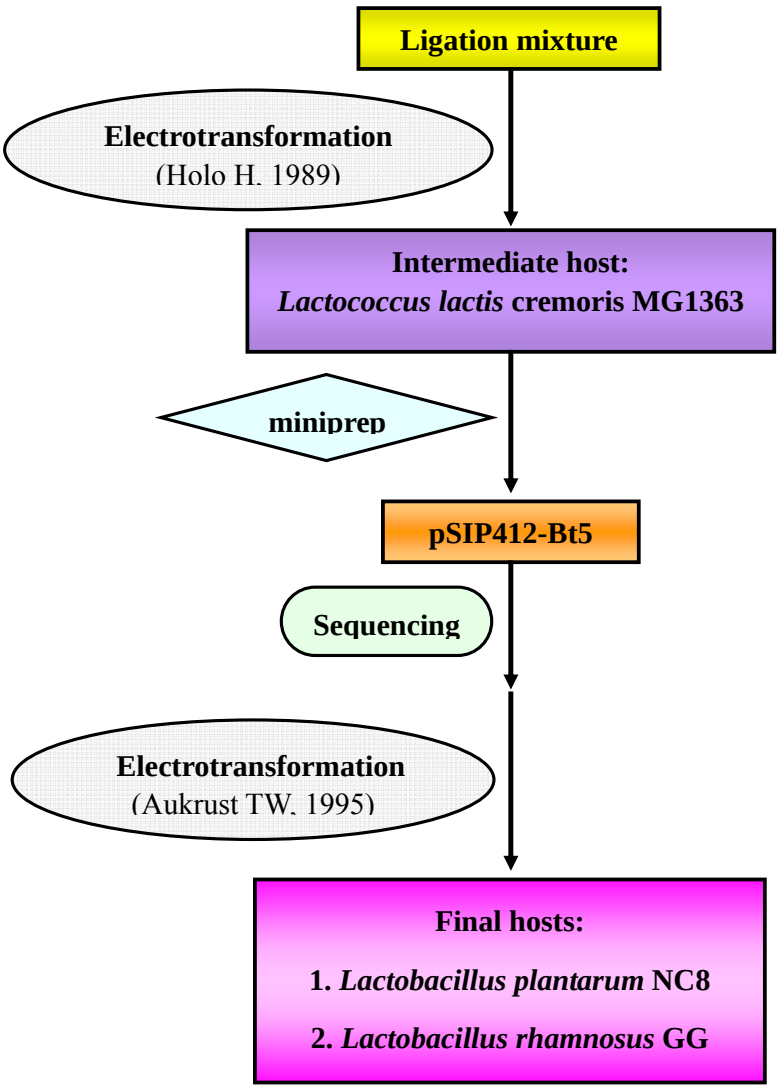


Figure 2.3 The schematic diagram showing the strategy for the generation of recombinant lactobacilli carrying pSIP412-Bt5. The ligation mixture was electrotransformed into the intermediate host *Lactococcus lactis* subspecies cremoris MG1363 for plasmid propagation. Small scale plasmid extraction was performed by miniprep and nucleotide sequences were confirmed by DNA sequencing. The pSIP412-Bt5 was finally electrotransformed into *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG for Blo t 5 expression.

2.2.7 Plasmid extraction and DNA sequencing

Small-scale plasmid extraction was performed by using Wizard® Plus SV miniprep DNA purification system (Promega, Madison, WI, USA) with little modification as described (O'sullivan DJ, 1993). Briefly, 3 ml overnight culture of recombinant *Lactococcus lactis* was centrifuged at 3,000 rpm for 10 mins. The bacterial pellet was resuspended in 250 μl of resuspension buffer containing 30 mg/ml of lysozyme and incubated at 37ºC for 20 mins. The subsequent steps followed the manufacturer's instructions. DNA sequencing was done by using the BigDye® Terminator v3.1 Cycle Sequencing R-100 Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendation.

2.2.8 Electrotransformation of *Lactobacillus* strains

Recombinant plasmid pSIP412-Bt5 was finally electroporated into *Lactobacillus plantarum* NC8 and LGG as described (Aukrust TW, 1995). Briefly, a single pulse of voltage, 1.5 kV; capacitance, 25 μF; resistance, 800 Ω was delivered. These settings gave a time constant of approximately 10 ms. Immediately following the pulse, bacteria were resuspended in MRSSM and further incubated at 30ºC for 2 hours. Transformants were selected by plating the bacteria on MRS agar plate containing 5 μg/ml of erythromycin. Transformants were visible after incubation at 30ºC for 2 to 3 days. The detailed recipe of culture media used for *Lactobacillus* strains was listed in Appendix 4.

2.2.9 The induction and quantification of Blo t 5 expression in recombinant lactobacilli

Recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were grown in MRS broth containing 5 μg/ml of erythromycin at 30ºC. When the OD_{600} reached 0.3, the Blo t 5 expression was induced by adding Sakacin P inducing peptide (*SppIP*) into the bacteria culture to a final concentration of 50 ng/ml (Sørvig E , 2005). The bacteria were cultured for additional 4 hours. A billion bacterial cells were harvested, washed with phosphate buffered saline (PBS) and finally resuspended in 200 μl of PBS. The bacterial counts were determined by OD_{600} where there is an estimation of 5 x 10^8 cfu/ml of bacteria at OD_{600} = 1. Bacterial cell suspensions were transferred into 1.5 ml tubes containing 0.1 g of 0.1 mm silica beads (Biospec Inc, Bartlesville, OK, USA). Cell lysates were prepared by vigorous shaking in a Bead-Beater (Biospec Inc, Bartlesville, OK, USA) with maximal speed for 90 secs. Twenty microliters of samples containing known colony forming units (cfu) of recombinant lactobacilli were separated on 7.5% SDS-PAGE and subjected to western blotting probed with ascites fluid at a dilution of 1:3. Ascites fluid was obtained from mice injected with hydridoma clones which produce mAb 4A7 as described (Yi FC, 2005). The gel image was captured using a densitometer LAS-3000 (Fujifilm, Stanford, CA). Each band density was measured in arbitrary units (AU unit) by using Multi Gauge general purpose analysis software (version 3.0). Different amounts (20, 40 and 80 ng) of recombinant yeast Blo t 5 were included and used as standard references. A

conversion graph of Blo t 5 amount (ng; y-axis) against band density (AU; x-axis) was plotted in order to estimate the amount of Blo t 5 expressed by recombinant *Lactobacillus plantarum* NC8 and recombinant LGG.

2.2.10 The Blo t 5 stability in recombinant LGG

The Blo t 5 expression in recombinant LGG was induced as described in section 2.2.9. Four hours after the induction of Sakacin P inducing peptide, 10 ml of bacterial cultures were harvested, washed with MRS broth and finally resuspended in 10 ml of MRS broth without Sakacin P inducing peptide. The bacteria were cultured for another 20 hours. 1 ml of bacterial culture was harvested at every hour up to 20 hours. Each sample containing one-tenth of bacterial cell lysates collected was separated on 7.5% SDS-PAGE and subjected to western blot probed with ascites fluid.

2.2.11 Preparation of live and heat-killed lactobacilli

Wildtype lactobacilli were grown in MRS broth whereas recombinant lactobacilli *were* grown in MRS broth containing 5 μg/ml erythromycin. When OD_{600} reached 0.3, the Blo t 5 expression was induced in recombinant lactobacilli by the addition of Sakacin P inducing peptide. The cultures of recombinant lactobacilli were incubated for additional 4 hours. The bacterial suspensions of wildtype lactobacilli and *SppIP*-induced recombinant lactobacilli were centrifuged at 3,000 rpm for 10 mins. For the preparation of live bacteria, lactobacilli were washed 2 times with

sterile PBS and finally resuspended in PBS to a final concentration of 5 x 10^{10} cfu/ml. For the preparation of heat-killed bacteria, lactobacilli were resuspended in PBS to a final concentration of 2 x 10^{9} cfu/ml. Heat-killed lactobacilli were prepared by heating the bacterial suspension in a 70ºC waterbath for 1 hour. The viability was determined by plating on MRS agar plates. No bacterial growth was detected after incubation at 37ºC for 72 hours.

2.2.12 Bone marrow-derived dendritic cells and bacteria coculture

Bone marrow cells were isolated and cultured as described with minor modifications (Lutz MB, 1999). Briefly, femora and tibiae of female 8-week-old C57BL/6 mice were removed and stripped of muscles and tendons (see Appendix 6). Both ends were cut. The marrow was flushed with complete RPMI 1640 medium using a 27-gauge needle. Cell suspension was centrifuged at 1300 rpm for 5 mins. Red blood cells were lysed by adding 1.5 ml of pre-warmed RBC lysis buffer (10 mM Tris, 0.83% NH_4Cl, pH 8.3) for 90 secs. Cells were washed 3 times with HBSS and finally resuspended in complete RPMI 1640 medium. The cell number was determined by using a hemocytometer.

Six million of cells were plated in Petri dish (92 mm x 17 mm; Nunc, Roskilde, Denmark) in 10 ml complete RPMI 1640 medium containing 40 ng/ml of GM-CSF (Peprotech, Rocky Hill, NJ). Cells were incubated at 37°C in a 5% CO_2 humidified incubator. On day 3, an additional 10 ml of freshly complete RPMI

medium containing 40 ng/ml of GM-CSF was added into each plate. On day 6, non-adherent cells were harvested by gentle pipetting. BMDCs were enriched by OptiPrep™ gradient centrifugation according to the manufacturer's recommendation (Axis Shield PoC AS, Oslo, Norway). Cells were washed 3 times with HBSS and finally resuspended in complete RPMI medium. The cell number was determined by using a hemocytometer.

A total of 1 x 10^6 BMDCs were plated in 1ml of complete RPMI medium in 6-well plate. BMDCs were co-cultured with wildtype or recombinant lactobacilli at DCs to bacteria (DCs:bacteria) ratio of 1:10 or 1:100 in the presence of 20 ng/ml of GM-CSF at 37°C in a 5% CO_2 humidified incubator. After 20-24 hours of stimulation, culture supernatants were collected for cytokine measurement by ELISA (section 2.2.23). The pulsed BMDCs were harvested for surface marker staining (section 2.2.13) or co-cultured with Blo t 5-specific T cells (section 2.2.14).

2.2.13 Surface marker staining of pulsed BMDCs

Pulsed BMDC were washed with PBS containing 1% BSA and subsequently labeled with fluorescence-conjugated mAbs at 4°C for 30 mins in 5 ml Falcon® polystyrene round-bottom tube (Becton Dickinson, JA, USA). Cells were washed 2 times with PBS containing 1% BSA and fixed with 1% paraformaldehyde. Flow cytometer was performed by using FACSCalibur and the data were analyzed by

using the CellQuest^{TM} software (Becton Dickinson, CA, USA). The following antibodies were used for surface marker staining: APC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse I-A^b, FITC-conjugated anti-mouse CD80, PE-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD40.

2.2.14 Co-culture of lactobacilli-pulsed BMDCs and Blo t 5-specific T cells

Pulsed BMDCs were co-cultured with 5 x 10^4 Blo t 5-specific T cells at a DC:T ratio of 0.2:1 or 1:1 in 96-well U-bottom microplate (Nunc, Roskilde, Denmark). Cultures were incubated at 37°C for 72 hours in a 5% CO_2 humidified incubator. Culture supernatants were collected for T cell cytokine measurement by ELISA (section 2.2.23). For the T cell proliferation assay, 1 μCi of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) was added into each well at 20 hours before cell harvest. On day 3, cells were harvested onto a glass fiber filter (Skatron instruments AS, Lier, Norway). After adding the scintillation fluid (Amersham Biosciences Corp), radioactivity was measured by multi-purpose scintillation counter LS 6500 (Beckman, Fullerton, CA, USA).

2.2.15 Flt3-derived dendritic cells

Bone marrow cells were obtained as previously described in section 2.2.12. Bone marrow cells were resuspended at 2 x 10^6 cells/ml in complete RPMI 1640 medium containing 200 ng/ml mouse Flt3L. 1mL of cells were plated in 6-well plate (Nunc, Roskilde, Denmark) and incubated at 37°C in a 5% CO_2 humidified

incubator for 9 days without disturbing. A total of 2 x 10^5 Flt3-derived dendritic cells were plated in 200µl of complete RPMI medium in a 96-well plate. These cells were co-cultured with wildtype and recombinant lactobacilli at DC to bacteria (DCs:bacteria) ratio of 1:10 or 1:100. After 20-24 hours of stimulation, culture supernatants were collected for cytokine measurement by ELISA (section 2.2.23).

2.2.16 Animal immunization protocols

2.2.16.1 Experiment I: *In vivo* immunogenicity study

Four-week-old female C57BL/6 mice were fed with 10^{10} cfu of wildtype or recombinant lactobacilli via a gavage needle for 3 consecutive days per week over 3 weeks. An additional control group of mice receiving 200 µl of PBS was included. Two days after the last feeding, the mice were sacrificed for T cell culture where cells were obtained from mesenteric lymph nodes and spleen.

2.2.16.2 Experiment II: Prophylactic model

Mice were fed with 10^{10} cfu of wildtype or recombinant lactobacilli as described in experiment I (section 2.2.16.1). An additional control group of mice receiving 200 µl of PBS was included. Sixteen days after the last feeding, all mice were intraperitoneally injected with 10 µg Blo t 5 in 200 µl PBS containing 4 mg alum. Three weeks apart, all mice received second intraperitoneal injection of 5 µg Blo t 5 in 200 µl PBS containing 4 mg alum. Two weeks later, mice were sacrificed.

Cells from mesenteric lymph nodes and spleen were collected for T cell culture.

2.2.16.3 Experiment III: Allergic airway inflammation model

Group of four mice were fed with 200 µl of PBS or 10^{10} cfu of wildtype or recombinant lactobacilli throughout the experiment. On day 3, a total of 2 x 10^6 Blo t 5-specific Th2 cells in 150 µl of PBS were adoptively transferred into each mouse via tail vein injection. All mice were intranasally challenged with 50 µg of Blo t 5 in 50 µl of PBS for the following three consecutive days. Three weeks apart, all mice were intranasally challenged with 50 µg of Blo t 5 in 50 µl of PBS for 3 consecutive days. On the next day, the bronchoalveolar lavage fluids (BALF) were collected.

2.2.17 Sera collection

Blood was collected via retro-orbital bleeding weekly. Sera were collected for the measurement of Blo t 5-specific antibodies by ELISA (section 2.2.21 and 2.2.22).

2.2.18 BALF collection and cytospin preparation

Mice were deeply anesthetized by intraperitoneal injection with 300 µl mixture containing 7.6 mg/ml ketamine and 0.1 mg/ml medetomidine. The trachea was exposed and cannulated by tracheostomy (20-gauge cannula). The lung was lavaged 3 times with 0.8ml of ice-cold HBSS. BALF was centrifuged at 5000 rpm for 5 mins. Cells were washed with HBSS and finally resuspended in 200 µl

RPMI 1640 medium for total cell count.

Cytospin was prepared by centrifugation of 1 x 10^5 cells in 100 µl of RPMI at 600 rpm for 5 mins by using Cytospin 3 (Shandon, Runcorn, Cheshire, UK). The cells were stained with Liu stain (Liu CH, 1953). Cells were identified and differentiated into the following groups: macrophages/monocytes (mac/mono), lymphocytes (lym), neutrophils (neu) and eosinophils (eos) based on the standard morphological techniques under the light microscope. Five hundred of cells were counted for each lavage sample. The percentage of each cell type was calculated.

2.2.19 Splenic and lymph nodes cell cultures

Single cell suspension was obtained by disrupting the spleen or lymph nodes in HBSS using 2 frosted slides. Red blood cells were lysed by adding 1.5 ml of pre-warmed RBC lysis buffer (10 mM Tris, 0.83% NH_4Cl, pH 8.3) for 90 secs. The cells were washed 3 times with HBSS and finally resuspended in complete RPMI 1640 medium. The cell number was determined by using a haemocytometer. To determine the cytokine production of freshly isolated splenic cells, a total of 4 x 10^5 splenocytes in 200 µl medium were stimulated in the presence of 10 µg/ml of Blo t 5 for 3 days in 96-well U bottomed plate (Nunc, Roskilde, Denmark). To determine the cytokine production of freshly isolated lymph nodes cells, 2 x 10^5 lymph node cells and 4 x 10^5 mitomycin C-treated antigen presenting cells (APCs) in 200 µl medium were stimulated in the presence

of 10 μg/ml Blo t 5 for 3 days. Culture supernatants were collected for cytokine measurement by ELISA (section 2.2.23).

2.2.20 Preparation of antigen presenting cells

Mitomycin-C treated splenocytes of naïve mice were used as APCs. Splenocytes were lysed with RBC lysis buffer, washed 3 times with HBSS and resuspended in PBS to a concentration of 5 x 10^7 cells/ml. Cells were treated with 50 μg/ml of Mitomycin-C in dark at 37°C waterbath for 20 mins. APCs were washed 3 times with HBSS and finally resuspended in complete RPMI medium.

2.2.21 Detection of Blo t 5-specific IgE and IgG1

The levels of Blo t 5 specific IgE and IgG1 were determined by Enzyme-Linked Immunosorbent Assay (ELISA). All samples were assayed in duplicates and 50 μl per well of reagents and samples were used unless otherwise stated. Briefly, 96-well EIA/RIA plates (Costar, Corning, NY, USA) were coated with 2 μg/ml purified anti-Igκ mAb or 5 μg/ml Blo t 5 in 0.1 M sodium bicarbonate (pH 8.3; United States Biological, Swampscott, MA, USA) at 4°C overnight. Plates were washed 3 times with TBS-T using the automated Columbus washer (TECAN, Austria) and blocked with 100 μl of blocking buffer (1% BSA in TBS-T) at room temperature for 1 hour. Plates were washed 3 times with TBS-T and added with diluted or undiluted samples. For quantification purposes, 2-fold serial dilutions of purified IgE or IgG1 were added to the anti-Igκ mAb-coated wells. Plates were

incubated at 4°C overnight. Plates were washed 3 times with TBS-T and incubated with biotinylated monoclonal rat anti-mouse IgE and IgG1 at room temperature for 1 hour. Plates were washed 3 times with TBS-T and incubated with alkaline-phosphatase-conjugated ExtraAvidin at a dilution of 1:2000 (Sigma, St. Louis, MO, USA) at room temperature for 1 hour. Finally, plates were washed 6 times with TSB-T and developed with Sigma Fast p-Nitrophenyl phosphatase substrate (pNPP; Sigma, St. Louis, MO, USA). Optical density was measured at 405 nm by an ELISA microplate reader (Tecan, Ges.m.b.H, Austria).

2.2.22 Mouse IgG2c quantitative ELISA

The amounts of Blo t 5-specific IgG2c was detected by using the mouse IgG2c ELISA Quantification Kit according to the manufacturer's instruction with minor modification. Briefly, 96-well EIA/RIA plates (Costar, Corning, NY, USA) were coated with 50 µl of 10 µg/ml purified goat anti-mouse IgG2c antibody or 5 µg/ml Blo t 5 in coating buffer (0.1 M $NaHCO_3$, pH 8.3) overnight at 4°C. Plates were washed 3 times with TBS-T using the automated Columbus washer (TECAN, Austria) and blocked with 100 µl of blocking buffer (1% BSA in TBS-T) for at least 1 hour at room temperature. Plates were washed 3 times with TBS-T and added with diluted or undiluted samples. For quantification purposes, serial dilutions of mouse sera with known concentration of IgG2c standard were included. Plates were incubated at room temperature for 2 hours. After washing the plates 3 times with TBS-T, 50 µl of HRP-conjugated goat anti-mouse IgG2c

was added into each well. The plates were incubated at room temperature for 1 hour. Plates were washed 6 times with TBS-T and signals were developed with 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) substrate (ABTS; Sigma, St. Louis, MO, USA). Optical density was measured at 405 nm by an ELISA microplate reader (Tecan, Ges.m.b.H, Austria).

2.2.23 Cytokine ELISA

All samples were assayed in duplicates and 25 µl per well of reagents and samples were used unless otherwise stated. Briefly, 96-well ELISA plates were coated with purified antibodies to mouse IL-4 (BVD4-1D11; 1 µg/ml), IL-5 (TRFK5; 2 µg/ml), IL-6 (MP5-20F3; 0.5 µg/ml), IL-10 (JES052A5; 4 µg/ml), IL-12 (48110.111; 2 µg/ml), IL-13(38213; 2 µg/ml), IFN-γ (R4-6A2; 2 µg/ml), TNF-α (AF-410-NA; 0.8 µg/ml) or TGF-β (A75-2.1; 2 µg/ml) in the coating buffer (0.1 M $NaHCO_3$, pH 8.3) and incubated at 4°C overnight. After washing 3 times with TBS-T, the plates were blocked with blocking buffer (1% BSA in TBS-T) at room temperature for 1 hour. Plates were washed 3 times with TBS-T and added with diluted or undiluted samples. For quantification purpose, recombinant mouse cytokines were prepared in 2-fold serial dilution in blocking buffer and included in each plate. The plates were incubated at 4°C overnight. The plates were washed 3 times with TBS-T and incubated with biotin-conjugated rat anti-mouse IL-4 (BVD6-24G2; 1.5 µg/ml), IL-5 (TRFK4; 2 µg/ml), IL-6 (MP5-32C11; 1 µg/ml), IL-10 (clone BAF417; 0.4 µg/ml), IL-12 (clone ; 2 µg/ml), IL-13 (clone BAF413;

0.2 μg/ml), IFN-γ (XMG1.2; 2 μg/ml), TNF-α (clone BAF410; 0.3 μg/ml) and TGF-β (A75-3.1; 2 μg/ml) at room temperature for 1 hour. Plates were washed 3 times with TBS-T and incubated with alkaline-phosphatase-conjugated ExtraAvidin (1:2000; Sigma, St. Louis, MO, USA) at room temperature for 1 hour. Finally, plates were washed 6 times with TBS-T and developed with Sigma Fast p-Nitrophenyl phosphatase substrate (pNPP; Sigma, St. Louis, MO, USA). Optical density was determined at 405 nm on an ELISA microplate reader (Tecan, Ges.m.b.H, Austria).

2.2.24 Statistical analysis

Experimental data were expressed as mean ± standard Error of Mean (SEM). Statistical analysis was conducted using Student's *t* test. A *P* value of less than 0.05 was taken as the level of significance.

Chapter 3

The *in vitro* characterisation of recombinant lactobacilli expressing Blo t 5

3.1 Introduction

In recent years, a substantial research effort has been devoted to the generation and modification of the expression vectors for the development of *Lactobacillus*-based vaccines for a number of diseases. To date, the commonly used inducible gene expression systems in lactobacilli are nisin-inducible expression vectors such as pNZ8037 (de Ruyter PG, 1996; Zhou XX, 2006; Daniel C, 2006; Rigaux P, 2009) and sakacin-inducible expression vectors such as pSIP400/500 series (Leer RJ, 1992; Axelsson L, 2003; Sørvig E, 2003; Mathiesen G, 2004; Sørvig E, 2005). Other expression vectors such as pLP400/500 vectors (Maassen CB, 1999; Pouwels PH, 2001; Ho PS, 2005; Kajikawa A, 2007; unpublished data our laboratory), pMEC vectors (Grangette C, 2001; Reveneau N, 2002; Grangette C, 2002) as well as pMD112 (Scheppler L, 2002; Scheppler L, 2005) have been evaluated and reported.

Our laboratory has previously generated recombinant *Lactobacillus casei* strain Shirota expressing house dust mite allergens such as Blo t 5 or Der p 2 by using pLP402 expression system. However, the antigen expression levels were generally very low (unpublished data). The poor antigen expression in these recombinant

lactobacilli represents a major limiting factor for the vaccine development. Thus, this study aims to explore the use of an inducible expression system for the generation of recombinant lactobacilli with high levels of antigen expression.

Lactic acid bacteria are known to produce two types of bacteriocins. The production of bacteriocins is often regulated via quorum-sensing mechanism based on a secreted peptide pheromone (Eijsink VGH, 2002; Quadri LE, 2002). The pheromone activates a two-component regulatory system consisting of a histidine kinase receptor and a cognate response regulator. Lantibiotics such as nisin is classified as class I bacteriocins. Lantibiotics itself acts as a pheromone that activates a two-component regulatory systems consisting of histidine kinase receptor and a cognate response regulator. Class II bacteriocins, such as sakacin A and sakacin P, produce and secrete a separate pheromone whose gene is usually co-transcribed with the genes encoding for the histidine kinase and the response regulator (Nes IF, 1999). In both cases, the activated response regulator enhances the transcription of all operons involved in the bacteriocin production.

The nisin-controlled expression (NICE) system has been developed, based on the autoregulatory properties of *Lactococcus lactis* nisin gene cluster, where the combination of the *nisA* promoter and the *nisRK* regulatory genes. NICE system is one of the best characterised inducible expression systems for the use in lactococci and lactobacilli. The expression of desired protein is induced by the externally

added nisin. It allows efficient overproduction of heterologous proteins in lactococci (de Ruyter PG, 1996) and lactobacilli (Kleerebezem M, 1997; Pavan S, 2000). However, it often exhibits significant basal activity and is poorly regulated in lactobacilli (Pavan S, 2000; Sørvig E, 2003).

Similar systems have been developed on the basis of genes and promoters that are involved in the production of class II bacteriocins (Leer RJ, 1992; Axelsson L, 2003; Sørvig E, 2003; Mathiesen G, 2004; Sørvig E, 2005). A series of "pSIP" expression vectors are constructed with a modular design that permits an easy exchange of all essential elements such as inducible promoter, cognate regulatory system, the gene of interest, antibiotics resistance marker and replicon (Sørvig E, 2003; Mathiesen G, 2004; Sørvig E, 2005). These vectors differ in their bacteriocins regulon, regulated promoter and the replicon. The levels of protein expression of different vectors have been tested (Sørvig E, 2005). Among them, pSIP412 vector has been constructed with high copy number, broad-host-range $SH71_{rep}$ replicon (de Vos WM, 1987), P_{orfX} promoter from sakacin P regulon and the cognate regulatory gene. The pSIP412 vector permits a controlled, considerable high-level expression of desired proteins in *Lactobacillus sakei* Lb790 and *Lactobacillus plantarum* NC8. This one-plasmid system not only keeps minimum background but also sustains the inducibility without integrating the genes into the host chromosome.

In the present study, pSIP412 was selected and evaluated for its inducible house dust mite allergen expression in *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG. The expression vector pSIP412 carrying the coding sequence of mature Blo t 5 was constructed and finally introduced it into *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG. The expression levels of Blo t 5 in recombinant *Lactobacillus plantarum* NC8 (rLp) and recombinant *Lactobacillus rhamnosus* GG (rLGG) were assessed by biochemical and densitometric methods. The immunomodulatory effects of these recombinant lactobacilli on dendritic cell maturation were assessed by phenotypic changes and cytokine production profiles of dendritic cells (DCs). In addition, the capability of recombinant lactobacilli-pulsed DCs to present antigen to antigen-specific T cells was examined by using a Blo t 5-specific T cell line.

3.2 Results

3.2.1 Purification of recombinant Blo t 5 from *Pichia pastoris* culture media

Recombinant Blo t 5 (rBlo t 5) protein was purified from the culture medium of the recombinant *Pichia pastoris* expressing Blo t 5 using the hydrophobic interaction and anion exchange chromatography as described in section 2.2.1. The purified yeast recombinant Blo t 5 protein was shown as a single band with an estimated molecular weight of about 14 kDa on a SDS-PAGE (Figure 3.1A). Western blot analysis showed that this band was specifically recognised by a Blo t

5-specific monoclonal antibody (mAb) 4A7 (Figure 3.1B). The estimated yield of recombinant Blo t 5 was estimated at 20 mg/L with the flask-culture method. The purified recombinant Blo t 5 protein was used for all *in vitro* and *in vivo* studies in this project.

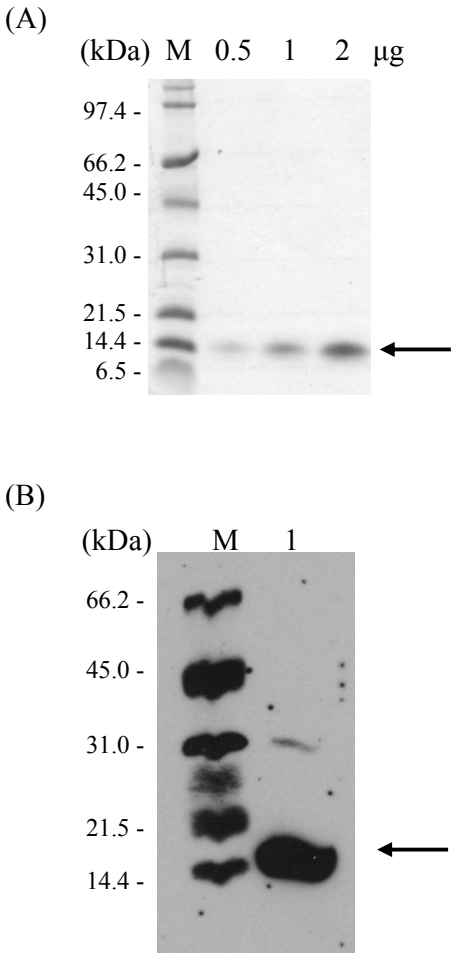


Figure 3.1 Characterization of recombinant Blo t 5 produced from *Pichia pastoris*. The purified recombinant Blot 5 protein was analyzed by SDS-PAGE. The amounts of protein loaded (µg) were indicated (A). The purified recombinant Blo t 5 was reacted with a monoclonal anti-Blo t 5 antibody 4A7 by Western blot (B). Arrows indicate the position of the recombinant Blo t 5 (~14 kDa). M: molecular mass markers (kDa); Lane 1: 0.5 µg of rBlo t 5.

3.2.2 Construction and transformation of pSIP412-Bt5 into lactobacilli.

The construction of recombinant plasmid pSIP412-Bt5 is schematically shown in Figure 2.1. As intact plasmids were necessary for successful transformation of most *Lactobacillus* strains (Maassen CB, 1999; Fuller R, 2000), *Lactococcus lactis* cremoris MG1363 was therefore used as an intermediate host for the plasmid propagation. The stepwise transformation procedures are summarised in Figure 2.2. Briefly, the pSIP412 plasmid DNA (8,405 bp) was digested with *NcoI*, treated with Klenow polymerase and further digested with *XhoI* to remove the aminopeptidase N (*pepN*) reporter gene (2,553 bp). The resulting plasmid (5,852 bp) was ligated with *XhoI*-digested cDNA encoding for mature Blot 5 (~351 bp). The pSIP412 plasmid DNA or ligation mixture was transformed into the immediate host *Lactococcus lactis* cremoris MG1363 (section 2.2.6). The pSIP412 and pSIP412-Bt5 plasmid DNA were extracted from the *Lactococcus lactis* cremoris MG1363 transformants and subsequently linearised with restriction enzyme *XhoI*. The fully linearized pSIP412 (~8.4 kb) and pSIP412-Bt5 (~6.2 kb) plasmid DNA were analysed on a 0.8% agarose gel (Figure 3.2A). Screening of the pSIP412-Bt5 containing *Lactococcus lactis* cremoris MG1363 transformants was performed by PCR method using the Blo t 5-specific primers (section 2.2.5). Recombinant plasmid pGEX-4T_1-Bt5 was included as a positive experimental control. The result showed that cDNA encoding the mature Blo t 5 was successfully inserted into the pSIP412-Bt5 (Figure 3.2B). The nucleotide sequences of the pSIP412-Bt5 construct were further confirmed by DNA

sequencing. Recombinant plasmid pSIP412-Bt5 was finally transformed into *Lactobacillus plantarum* NC8 and *Lactobacillus rhamnosus* GG (LGG) by electrotransformation (section 2.2.8).

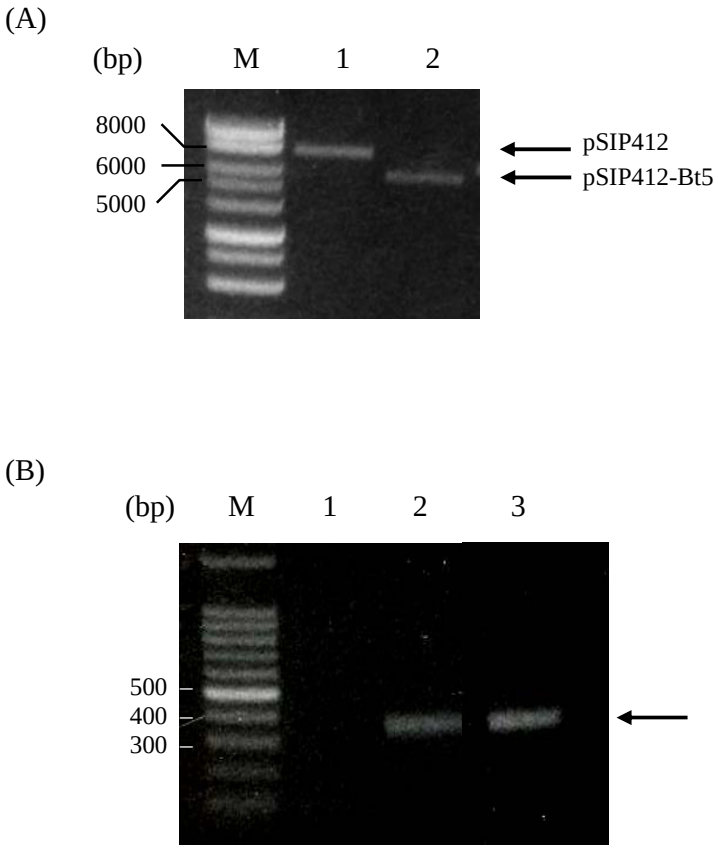


Figure 3.2 Analysis of pSIP412-Bt5 construct. (A) pSIP412 (8,405 bp) and pSIP412-Bt5 (6,208 bp) which were extracted from the intermediate host *Lactococcus lactis* subspecies cremoris MG1363 were digested with *Xho*I. Arrows indicate the bands of linearized pSIP412 and pSIP412-Bt5. (B) Polymerase chain reaction amplification of Blo t 5 coding sequence from extracted plasmids by using the Blo t 5 specific primers. Arrow indicates the band of the Blo t 5 coding fragment (~351 bp). M: hundred base pair (bp) DNA ladders; Lane 1, pSIP412 extracted from *Lactococcus lactis*; Lane 2, pSIP412-Bt5 extracted from *Lactococcus lactis*; Lane 3, pGEX-4T$_1$-Bt5 extracted from *Escherichia coli*.

3.2.3 Expression kinetics of Blo t 5 in recombinant lactobacilli

The Blo t 5 expression in recombinant *Lactobacillus plantarum* NC8 and recombinant LGG was induced by an addition of Sakacin P inducing peptide (*SppIP*) to bacterial cultures when the OD_{600} reached 0.3. The protein expression kinetics of Blo t 5 in recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were monitored using samples taken at OD_{600} of 0.6, 1.2 and 2.0. The results were shown in Figure 3.3A and 3.3B, respectively. The expression levels of Blo t 5 in both recombinant lactobacilli increased in a time-dependent manner. Notably, the level of Blo t 5 expressed in recombinant *Lactobacillus plantarum* NC8 was relatively higher than that of the recombinant LGG.

To further confirm the expression of Blo t 5 in recombinant *Lactobacillus plantarum* NC8 and recombinant LGG, western blot was performed by using ascites fluid containing mAb 4A7 (Yi FC, 2005). As shown in Figure 3.4, mAb 4A7 reacted strongly with a protein of molecular weight of about 14 kD in both recombinant *Lactobacillus plantarum* NC8 and recombinant LGG, confirming that a monomeric form of Blo t 5 protein was indeed successfully expressed in these recombinant lactobacilli. A weak band with an estimated size of 28 kDa was also observed in the *SppIP*-induced culture of recombinant *Lactobacillus plantarum* NC8. This band may represent a dimer of Blo t 5. Uninduced culture samples of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG showed no reactivity towards mAb 4A7.

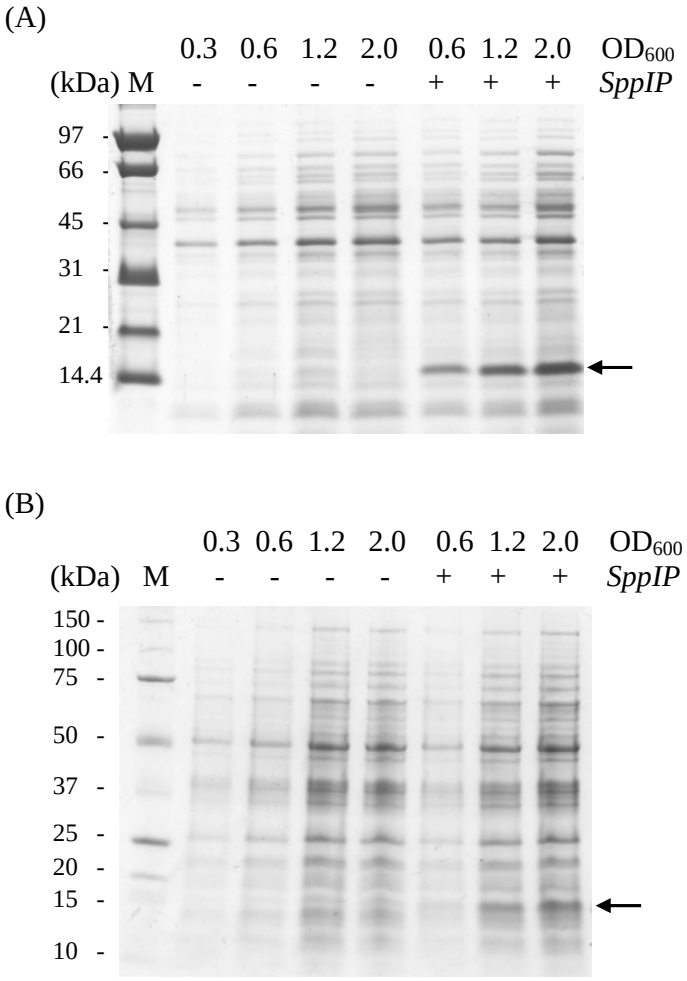


Figure 3.3 Kinetics of Blo t 5 expression in recombinant lactobacilli. Recombinant Blo t 5 expressed in *Lactobacillus plantarum* (A) and *Lactobacillus rhamnosus* GG (B) were analyzed by Coomassie blue-stained SDS-PAGE gel. Blo t 5 expression was induced by Sakacin P inducing peptide (*SppIP*) when OD_{600} reached 0.3. After the addition of Sakacin P inducing peptide, cultures of recombinant lactobacilli were sampled at indicated OD_{600} values. The cultures of recombinant lactobacilli without Sakacin P inducing peptide were included as controls. The gel loading of each lane was corresponding to 100 µl of bacterial culture. Arrows indicate the position of recombinant Blo t 5 (~14 kDa). M: molecular mass markers (kDa).

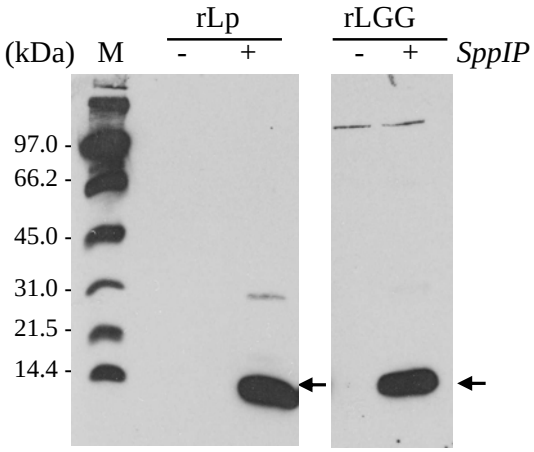


Figure 3.4 Western blot analysis of Blo t 5 expressed in recombinant lactobacilli. Recombinant Blo t 5 expressed in *Lactobacillus plantarum* (rLp) and *Lactobacillus rhamnosus* GG (rLGG) were analyzed by probed with monoclonal anti-Blo t 5 antibody 4A7. Blo t 5 expression was induced by Sakacin P inducing peptide (*SppIP*) when the OD_{600} reached 0.3. After 4 hours, 1 ml of cultures were collected from the induced and the non-induced cultures of recombinant lactobacilli. The gel loading of each lane was corresponding to 100 µl of bacterial culture. Arrows indicate the position of Blo t 5 (~14 kDa). M: molecular mass markers (kDa).

3.2.4 Quantification of Blo t 5 in recombinant lactobacilli

To quantitate the amount of Blo t 5 expressed by recombinant *Lactobacillus plantarum* NC8 and recombinant LGG, western blot and densitometric analysis were performed (Figure 3.5A). Three doses of cell lysates containing the indicated colony formation units (cfu) of recombinant *Lactobacillus plantarum* NC8 ($1x10^6$, $2x10^6$ and $3x10^6$ cfu) and recombinant LGG ($2x10^6$, $4x10^6$ and $8x10^6$ cfu) were used for western blot analysis with ascites fluid. Densitometric analysis was performed on gel image captured using LAS-3000. The band density was measured as arbitrary unit (AU). Based on the known amounts (20, 40 and 80 ng) of rBlo t 5, a reference curve of Blo t 5 quantities (y-axis) versus AU (x-axis) was constructed to convert the amounts of Blo t 5 expressed in recombinant lactobacilli (Figure 3.5B). The estimated amounts of Blo t 5 expressed by 10^{10} cfu of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were approximately 400 and 150 µg, respectively (Table 3.1). The amount of Blo t 5 expressed by recombinant LGG was about 2.5-fold lower than that of the recombinant *Lactobacillus plantarum* NC8. The lower level of protein expression in recombinant LGG could be due to a number of factors such as mRNA stability, codon usage of the protein, host cell physiology and potential negative effects of the protein on the host cell (Sorvig E, 2005). A further experiment was carried out to determine whether the lower level of Blo t 5 expression was correlated to poor protein stability. In this experiment, the Blo t 5 expression in recombinant LGG was induced by Sakacin P. After 4-hour of induction, bacteria were washed and

grown in MRS broth without Sakacin P for another twenty hours. Western blot analysis showed that Blo t 5 was stable in recombinant LGG for at least up to twenty hours (Figure 3.6).

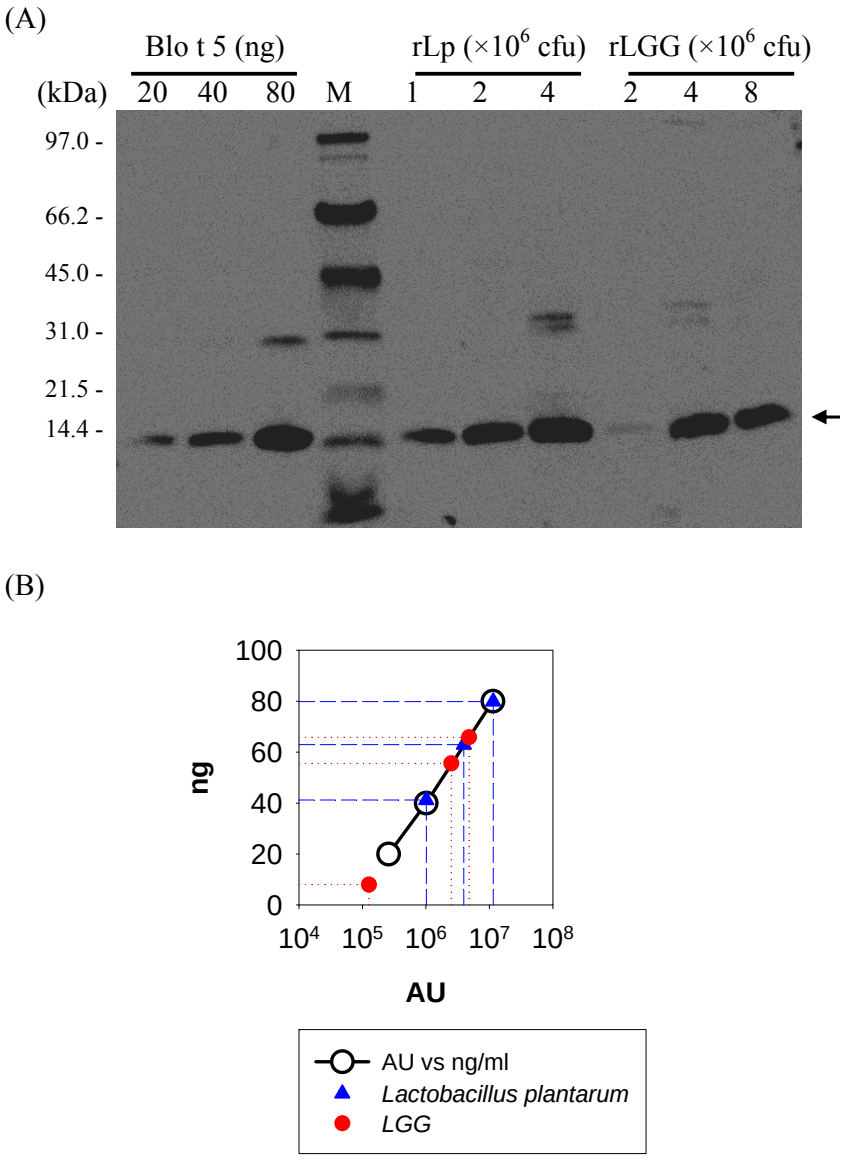


Figure 3.5 The quantification of Blo t 5 expression in recombinant lactobacilli. (A) Cell lysates containing the indicated colony formation units (cfu) of recombinant *Lactobacillus plantarum* NC8 (rLp) and recombinant *Lactobacillus rhamnosus* GG (rLGG) were separated in 7.5% SDS-PAGE and further analyzed by Western blot. Arrows indicate the position of Blo t 5 (~14 kDa). The purified Blo t 5 protein was used as standards for the indicated amounts. The gel image was captured by the FujiFilm LAS-3000. The band density was measured and expressed as AU units. M: molecular mass markers (kDa). (B) A reference curve of Blo t 5 quantities (y-axis) versus AU units (x-axis) was used to convert the amounts of Blo t 5 expressed in recombinant *Lactobacillus plantarum* NC8 (▲) and recombinant *Lactobacillus rhamnosus* GG (●).

Table 3.1 The amount of Blo t 5 expressed in recombinant *Lactobacillus* strains.

Bacteria strains	Blo t 5 amount / 1×10^{10} cfu
Recombinant *Lactobacillus plantarum* NC8 (rLp)	400 μg
Recombinant *Lactobacillus rhamnosus* GG (rLGG)	150 μg

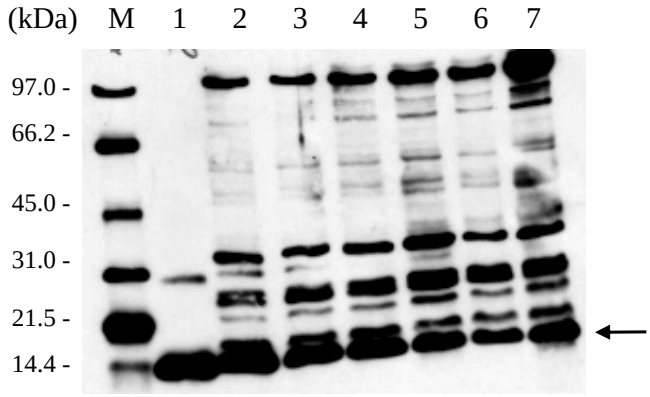


Figure 3.6 The stability of Blo t 5 produced in *Lactobacillus rhamnosus* GG. Blo t 5 expression was induced by Sakacin P inducing peptide (*SppIP*) when the OD_{600} reached 0.3. After 4-hour of induction, the bacteria were washed and cultured in MRS broth without Sakacin P inducing peptide for 20 hours. Samples of 1 ml of bacterial cultures were harvested at 1 hour (lane 2), 2 hours (lane 3), 3 hours (lane 4), 4 hours (lane 5), 5 hours (lane 6) and 20 hours (lane 7) after withdrawing the Sakacin P inducing peptide from the culture medium. Bacterial cell lysates were separated on 7.5% SDS-PAGE and subjected to Western blotting probed with monoclonal anti-Blo t 5 antibody 4A7. The gel loading of each lane was corresponding to 100 µl of the bacterial culture. Arrows indicate the position of Blo t 5 (~14 kDa). Lane 1: 150 ng of purified recombinant Blo t. M: molecular mass markers (kDa).

3.2.5 The immunomodulatory effect of recombinant lactobacilli on murine bone marrow-derived dendritic cells (BMDCs)

To determine the immunomodulatory effects of recombinant lactobacilli on BMDCs, the maturation of BMDC was assessed by the surface marker expression and cytokine production profiles. In this experiment, BMDCs were co-cultured with recombinant *Lactobacillus plantarum* NC8 or recombinant LGG at DCs:bacteria ratio of 1:10 or 1:100 for 20-24 hours. Unpulsed BMDCs, BMDCs pulsed with 1 μg/ml of lipopolysaccharide (LPS) or 10 μg/ml of Blo t 5 or wildtype lactobacilli or wildtype lactobacilli plus Blo t 5 were included as controls.

3.2.5.1 The expression profiles of surface markers on BMDCs

Pulsed BMDCs were stained with specific antibodies for CD86, MHC class II I-A^b, CD40, CD80 and CD11c for flow cytometric analysis. As compared to the unpulsed BMDCs, LPS, but not the exogeneous Blo t 5 protein, induced the maturation of BMDCs as shown by the upregulation of CD86, MHC class II I-A^b, CD40 and CD80 expression (Figure 3.7A). At both DCs:bacteria ratios of 1:10 and 1:100, all the lactobacilli-pulsed DCs displayed similar surface expression profiles of MHC class II I-A^b, CD40 and CD80 with the exception of CD86 (Figure 3.7B, 3.7C, 3.7D and 3.7E). The expression of the CD86 molecules was further upregulated when the DCs:bacteria ratio was raised from 1:10 to 1:100. No differences in the surface marker expression profiles of BMDCs were observed

when BMDCs were pulsed with wildtype lactobacilli alone or in combination with exogeneous Blo t 5 protein (data not shown).

3.2.5.2 The cytokine production by BMDCs

The levels of cytokines produced by pulsed BMDCs in the culture supernatants were measured by ELISA. The results showed that LPS-pulsed BMDCs produced significant levels of IL-10, IL-12, IL-6 and TNF-α whereas the unpulsed BMDCs only produced low basal levels of these cytokines (Figure 3.8). Interestingly, significant high levels of IL-10, IL-12, TNF-α and IL-6 were produced by the recombinant LGG-pulsed BMDCs as compared to that of the recombinant *Lactobacillus plantarum* NC8-pulsed BMDCs at DCs:bacteria ratio of 1:10 or 1:100. The level of IL-10 was significantly higher in wildtype LGG-pulsed BMDCs than recombinant LGG- and wildtype *Lactobacillus plantarum* NC8-pulsed BMDCs at both DCs:bacteria ratios (Figure 3.8A). On the other hand, the level of IL-12 was significantly higher in both recombinant lactobacilli-pulsed BMDCs than that of the both wildtype lactobacilli-pulsed BMDCs at 1:100 DCs:bacteria ratio (Figure 3.8B). The levels of TNF-α and IL-6 produced by all lactobacilli-pulsed BMDCs were higher than unpulsed BMDCs (Figure 3.8C and 3.8D).

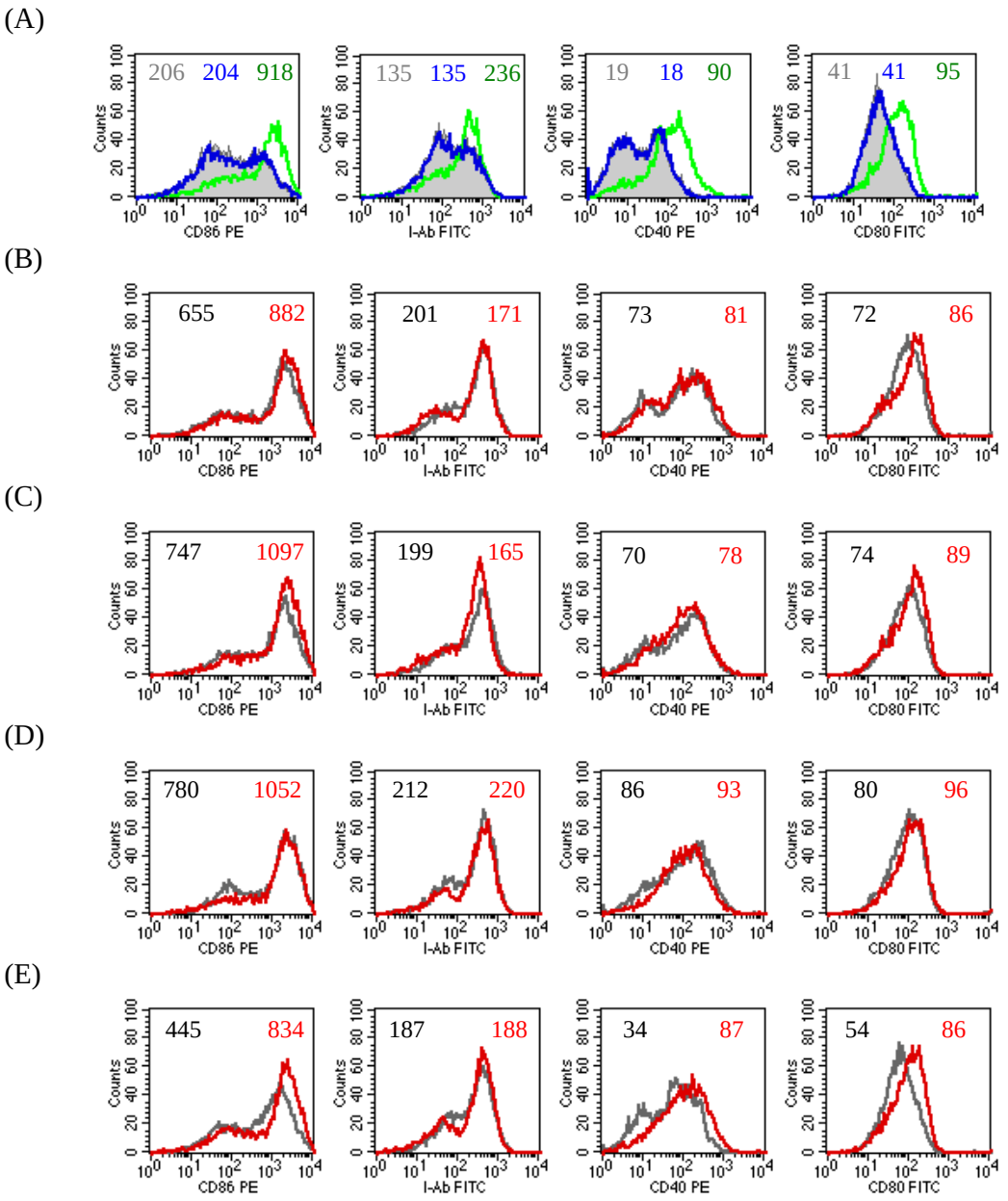


Figure 3.7 The phenotypes and maturation status of murine bone-marrow derived dendritic cells (BMDCs) co-cultured with recombinant lactobacilli. The immature BMDCs were co-cultured with wildtype or recombinant lactobacilli for 20-24 hours and the cell surface markers CD86, MHC class II I-A^b, CD40, CD80 and CD11c were analyzed with flow cytometry. DCs with medium alone (filled grey histograms), LPS-stimulation (open green histograms) and Blo t 5-stimulation (open blue histograms) were included as references (A). 1 x 10^6 of BMDCs were stimulated with wildtype *Lactobacillus plantarum* (B), recombinant *Lactobacillus plantarum* (C), wildtype *LGG* (D) and recombinant LGG (E) at the DCs:bacteria ratio of 1:10 (open grey histograms) or 1:100 (open red histograms). The numbers on top of each histogram represent the geometric means.

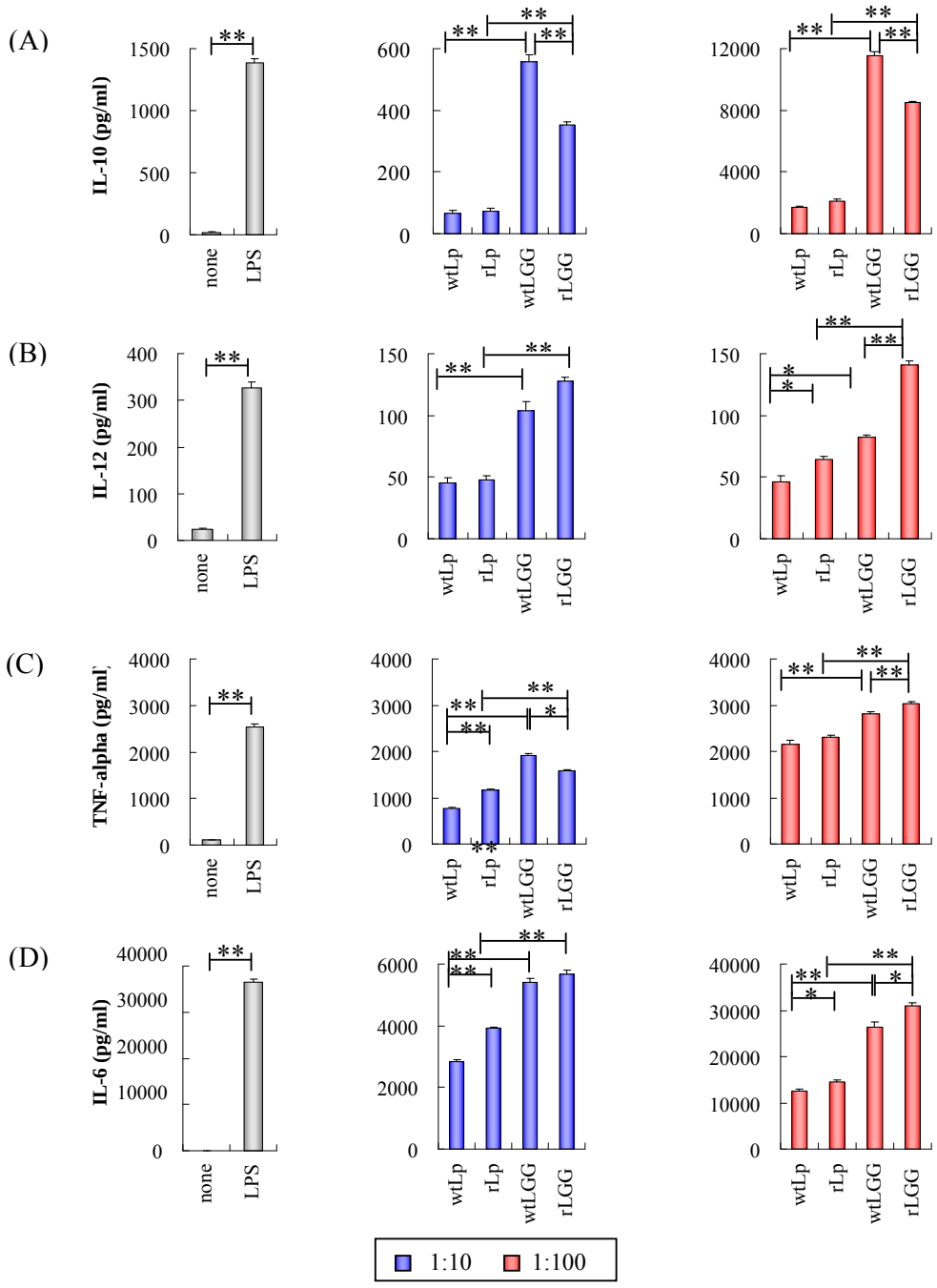


Figure 3.8 The cytokine production of murine bone-marrow derived dendritic cells (BMDCs) co-cultured with recombinant lactobacilli. 1 x 10^6 of BMDCs were co-cultured with recombinant *Lactobacillus plantarum* (rLp) or recombinant *Lactobacillus rhamnosus* GG (rLGG) at the DCs:bacteria ratio of 1:10 (■) or 1:100 (■). Medium alone (none), lipopolysaccharide (LPS) and wildtype lactobacilli (wtLp or wtLGG) were included as controls. Culture supernatants were collected after 20-24 hours of incubation. The levels of IL-10 (A), IL-12 (B), TNF-α(C) and IL-6 (D) were measured by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$.

3.2.6 The effects of recombinant lactobacilli on cytokine production by murine Flt3-derived dendritic cells

The immunomodulatory effects of recombinant lactobacilli on Flt3-derived DCs were evaluated. LPS- and lactobacilli-pulsed Flt3-derived DCs were capable of producing substantial amounts of IL-10, IL-12, IL-6 and TNF-α, but the levels of IL-10 and IL-6 production were relatively lower as compared to that of BMDCs (Figure 3.9). Unlike the BMDCs, Flt3-derived DCs pulsed with lactobacilli did not induce IL-6 production at DCs:bacteria ratio of 1:10 (Figure 3.9D). Notably, the IL-10 levels produced by recombinant *Lactobacillus plantarum* NC8-pulsed Flt3-derived DCs were significantly lower than that of the wildtype *Lactobacillus plantarum* NC8- and recombinant LGG-pulsed Flt3-derived DCs (Figure 3.9A). The IL-12 level produced by recombinant *Lactobacillus plantarum* NC8-pulsed Flt3-derived DCs was only found significantly lower than that of the wildtype *Lactobacillus plantarum* NC8- and recombinant LGG -pulsed Flt3-derived DCs at a DCs:bacteria ratio of 1:100 (Figure 3.9B). As compared to the wildtype *Lactobacillus plantarum* NC8-pulsed Flt3-derived DCs, wiltype LGG-pulsed Flt3-derived DCs produced significant high levels of IL-10 and IL-12 at DCs:bacteria ratio of 1:100 (Figure 3.9A and 3.9B).

Taken together, the levels of all cytokines tested in BMDCs and Flt3-derived DCs were profoundly higher in LGG strain than *Lactobacillus plantarum* NC8 strain. These findings implied that LGG and *Lactobacillus plantarum* NC8 possess

distinct adjuvant properties and induce differential immunomodulation on BMDCs as well as Flt3-derived DCs.

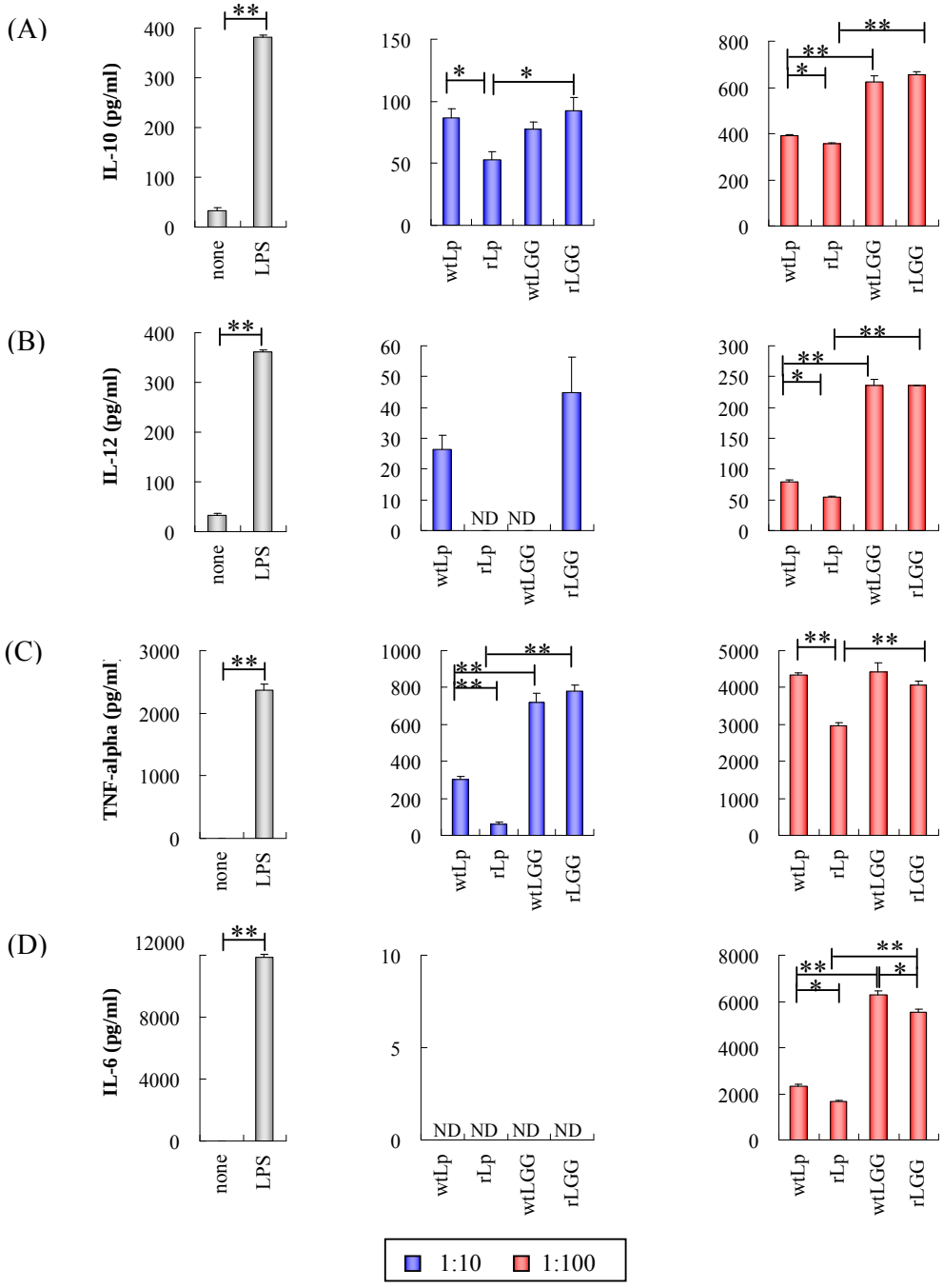


Figure 3.9 The cytokine production of murine Flt3-derived dendritic cells co-cultured with recombinant lactobacilli. 2 x 10^5 of Flt3-derived DCs were co-cultured with recombinant *Lactobacillus plantarum* (rLp) or recombinant *Lactobacillus rhamnosus* GG (rLGG) at the DCs:bacteria ratio of 1:10 (■) or 1:100 (■). Medium alone (none), lipopolysaccharide (LPS) and wildtype lactobacilli (wtLp or wtLGG) were included as controls. Culture supernatants were collected after 20-24 hours of incubation. The levels of IL-10 (A), IL-12 (B), IL-6 (C) and TNF-α (D) were measured by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$; ND: non-detectable.

3.2.7 Induction of antigen-specific T cell activation by recombinant lactobacilli pulsed-murine BMDCs

In order to determine whether the recombinant lactobacilli-pulsed BMDCs can serve as competent antigen presenting cells (APCs) to induce the antigen-specific T cell activation, Blo t 5-specific T cells were co-cultured with recombinant lactobacilli-pulsed BMDCs and the T cell proliferation as well as the cytokine production by these specific T cells were assessed. In this experiment, BMDCs were pulsed with recombinant *Lactobacillus plantarum* NC8 and recombinant LGG at DCs:bacteria ratio of 1:10 or 1:100 for 20-24 hours. Pulsed BMDCs were harvested and co-cultured with Blo t 5-specific T cells at DC:T ratio of 0.2:1 or 1:1 for 3 days. Unpulsed, LPS-, Blo t 5-, wildtype lactobacilli- and wildtype lactobacilli plus exogeneous Blo t 5-pulsed BMDCs were included as controls.

3.2.7.1 The proliferation of Blo t 5-specific T cells

Under the culture condition of 1:1 DC:T ratio, Blo t 5-pulsed BMDCs, but not the unpulsed BMDCs, induced Blo t 5-specific T cell proliferation (Figure 3.10A). Notably, BMDCs pulsed with the wildtype lactobacilli plus exogeneous Blo t 5- and recombinant lactobacilli strongly induced Blo t 5-specific T cell proliferation that was at least three-fold higher than that of the Blo t 5-pulsed BMDCs. Wildtype lactobacilli- and LPS-pulsed BMDCs failed to induce any Blo t 5-specific T cell proliferation. Under the culture condition of 0.2:1 DC:T ratio,

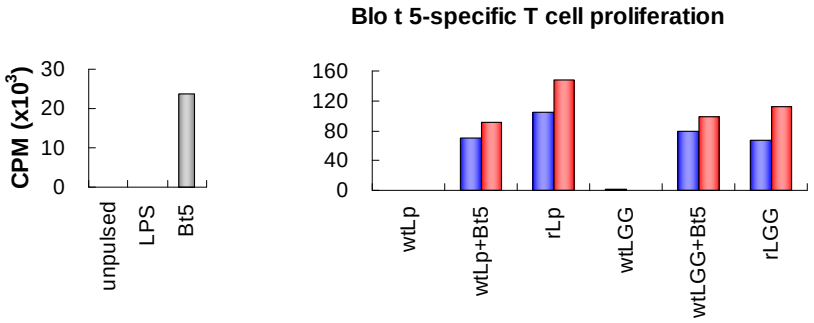
similar results were obtained with reduced levels of T cell proliferation (Figure 3.10B).

3.2.7.2 The cytokine production by Blo t 5-specific T cells

The Blo t 5-specific T cells used in this study are Th2-type cells. Upon the activation of Blo t 5 protein, these specific T cells are capable of producing signature Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13. Blo t 5-pulsed BMDCs, but not the unpulsed BMDCs, induced the cytokine production by Blo t 5-specific T cells (Figure 3.11). Under the culture condition of 1:1 DC:T ratio, wildtype lactobacilli plus exogenous Blo t 5- and recombinant lactobacilli-pulsed BMDCs led to higher cytokine production by Blo t 5-specific T cells. Wildtype lactobacilli- and LPS-pulsed BMDCs failed to induce cytokine production by these specific T cells. Similar experiments performed at DC:T ratio of 0.2:1 gave similar results with reduced levels of cytokine production by the activated T cells (data not shown).

These data clearly demonstrated that murine BMDCs could efficiently uptake the Blo t 5-expressing recombinant lactobacilli, process and subsequently present Blo t 5 to the Blo t 5-specific T cells as indicated by the high levels of T cell proliferation and cytokine production by these Blo t 5-specific T cells.

(A)



(B)

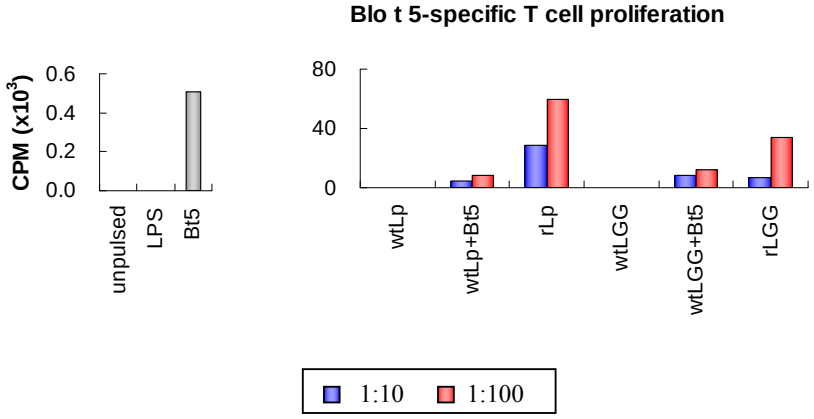


Figure 3.10 Recombinant lactobacilli-pulsed bone-marrow derived dendritic cells (BMDCs) induced the proliferation of a Blo t 5-specific T cells. BMDCs were pulsed with recombinant *Lactobacillus plantarum* (rLp) or recombinant *Lactobacillus rhamnosus* GG (rLGG) at the DCs:bacteria ratio of 1:10 (■) or 1:100 (■) for 20-24 hours. Unpulsed-, lipopolysaccharide (LPS)-, Blo t 5-, wildtype lactobacilli (wtLp or wtLGG)- and wildtype lactobacilli plus Blo t 5 (wtLp+Bt5 or wtLGG+Bt5)-pulsed DCs were included as controls. Different stimuli-pulsed BMDCs were harvested, washed and co-cultured with a Blo t 5-specific T cells (5 x 10^4 per well) at the DCs:T cells ratio of 1:1 (A) or 0.2:1 (B). Blo t 5-specific T cell proliferation was measured by the [^{3}H]-thymidine incorporation on day 3. Each well was added with 1 μCi of [^{3}H]-thymidine for the last 20 hours of culture. cpm, counts per minute.

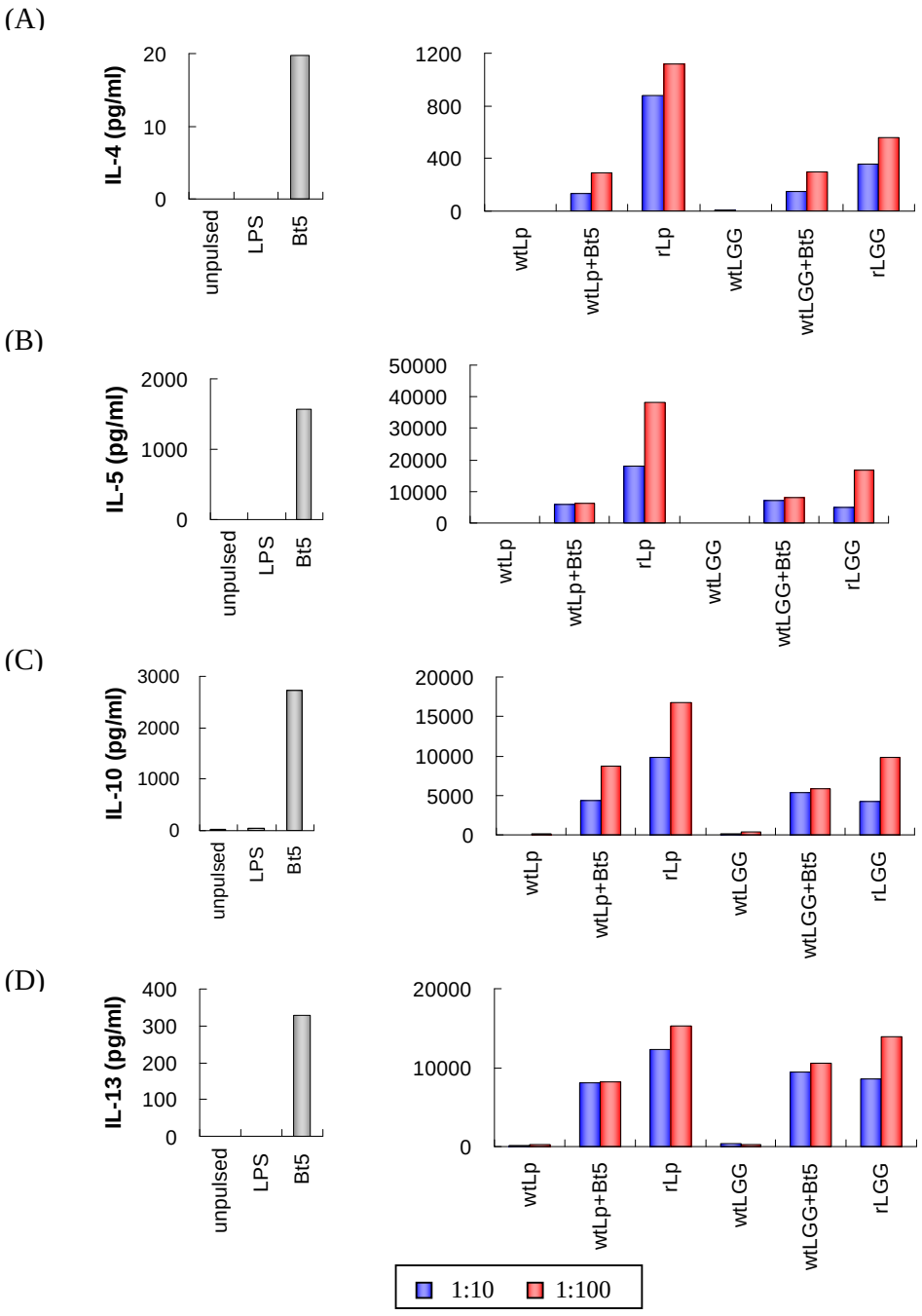


Figure 3.11 Recombinant lactobacilli-pulsed bone-marrow derived dendritic cells (BMDCs) induced the cytokine production of Blo t 5-specific T cells. BMDCs were pulsed with recombinant *Lactobacillus plantarum* (rLp) or recombinant *Lactobacillus rhamnosus* GG (rLGG) at the DCs:bacteria ratio of 1:10 (■) or 1:100 (■) for 20-24 hours. Unpulsed-, lipopolysaccharide (LPS)-, Blo t 5-, wildtype lactobacilli (wtLp or wtLGG)- and wildtype lactobacilli plus Blo t 5 (wtLp+Bt5 or wtLGG+Bt5)-pulsed DCs were included as controls. Pulsed BMDCs were harvested, washed and co-cultured with a Blo t 5-specific T cells (5 x 10^4 per well) at the DCs:T cells ratio of 1:1. Culture supernatants were collected on day 3 for the measurement of IL-4 (A), IL-5 (B), IL-10 (C) and IL-13 (D) by enzyme-linked immunosorbent assays.

3.3 Discussion

For the development of an effective antigen-expressing *Lactobacillus*-based oral vaccine, several parameters such as the choice of *Lactobacillus* strain, the amount and localisation of antigen expressed have to be taken into consideration. Firstly, the proper selection of *Lactobacillus* strains is crucial. Although dietary *Lactobacillus* strains with GRAS status are generally safe for human oral consumption, different *Lactobacillus* strains possess differential intrinsic adjuvanicity (Maassen CB, 2000). They differ in their cell-wall components, soluble factors, genomic DNA, physiology and behavior in the gastrointestinal tract, thereby affecting their immunogenicity and the induction of systemic immune responses. Previous published studies have suggested that *Lactobacillus plantarum* could be a potent Th1 inducer (Murosaki S, 1998; Hessle C, 1999; Repa A, 2003) whereas LGG could be a potent Treg inducer (Feleszko W, 2007). Based on their reported differential intrinsic adjuvant properties, the side by side comparative studies on recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were performed to evaluate their potential exploitation as a live vector for antigen delivery as well as an adjuvant to enhance antigen-specific adaptive immunity. This study paves the way for the development of an antigen-based oral vaccine for allergic diseases such as asthma.

In addition to the choice of *Lactobacillus* strains, the cellular location of the antigen expressed affects the immunogenicity of the recombinant lactobacilli. The

intracellular production of protein might be more efficient in the induction of specific immune responses than the cell surface-anchored protein (Norton PM, 1996). The oral delivery of tetanus toxin fragment C (TTFC) expressed as an intracellular antigen was more effective than cell-surface expression (Shaw DM, 2000). Conversely, cell surface-anchored antigen has been reported to be more immunogenic than intracellularly expressed antigen and secreted antigen (Dieye Y, 2003; Bermúdez-Humarán LG, 2004). However, the surface antigen expression may be more susceptible to low pH, bile acid or proteolytic environments encountered by vaccine vectors following mucosal immunisation. Therefore, the best location of an expressed antigen for an optimal mucosal immunisation remains unclear and deserves more studies.

Depending on the immunogenicity of the antigen and the immunisation regime, a specific threshold for the amount of expressed antigen is required to elicit an immune response (Zegers ND, 1999; Grangette C, 2001; Grangette C, 2002; Reveneau N, 2002; Seegers JF, 2002; Adel-Patient K, 2005; Wells JM, 2008). It has been reported that no specific antibody response against protective antigen (PA) of *Bacillus anthracis* was detected probably due to the low intracellular production of PA by using pLP503 expression vector (Zegers ND, 1999). In addition, recombinant *Lactobacillus* strains expressing TTFC failed to elicit specific antibody response (Grangette C, 2001; Grangette C, 2002). Reveneau N et al. has demonstrated that the highest specific antibody levels were obtained

with the strains producing high intracellular levels of TTFC (Reveneau N, 2002). These findings suggested that the expression level of the intracellular protein is critical in the induction of antigen-specific immune responses.

This study was the first to describe the use of Sakacin P-inducible expression vector pSIP412 in *Lactobacillus plantarum* NC8 or LGG as a host for the expression of a major house dust allergen, Blo t 5. It is well established that the Sakacin P-inducible expression system could produce high levels of tested antigens in *Lactobacillus plantarum* NC8 (Sørvig E, 2005), but there is no published report on the evaluation of this expression system in LGG to date. Therefore, the main focus of this study aimed to perform a systematic comparative evaluation of this expression vector system in two *Lactobacillus* stains. The expression level of Blo t 5 in recombinant LGG was nearly 2.7-fold lower than in recombinant *Lactobacillus plantarum* NC8 (Table 3.1). Notably, Blo t 5 was expressed stably in the recombinant LGG up to twenty hours post-induction (Figure 3.6). With the use of nisin-inducible pNZ8037 expression system, Daniel C et al. has reported that 10^{10} cfu of recombinant *Lactococcus lactis* and recombinant *Lactobacillus plantarum* expressed 4 and 16 μg of Bet v1, respectively. In the current study, the amount of Blo t 5 antigen expressed was at least 10-fold higher by using *SppIP*-inducible pSIP412 expression system as compared to the nisin-inducible pNZ8037 expression system (Daniel C, 2006). In addition, Kajikawa A et al. has reported that 10^{10} cfu of recombinant *Lactococcus*

casei expressing less than 5 μg of flagellin failed to induce antigen-specific antibody production (Kajikawa A, 2007). Furthermore, the amounts of Blo t 5 expressed in recombinant *Lactobacillus* plantarum NC8 and recombinant LGG by using pSIP412 expression vector were much higher than that using pLP402 expression vector (unpublished data from our laboratory). These findings suggested that pSIP412 represents a better expression vector system for Blo t 5 antigen expression in both *Lactobacillus* strains, therefore greatly facilitating further development of the *Lactobacillus*-based oral vaccine for allergic diseases.

Dendritic cells are professional antigen presenting cells (APCs) that prime and direct the differentiation of naïve T cells into Th1, Th2 or Treg cell subsets (Banchereau J, 1998). DC maturation is necessary for both Th1 and Th2 cell differentiation (Kaliński P, 1999) and T cell tolerance requires certain degree of DC maturation (Perez VL, 1997; Albert ML, 2001; Figdor CG, 2004). The DC-derived cytokines is the most important factor in shaping the T cell responses (Mazzoni A, 2004). IL-12-producing DCs promotes the polarisation of IFN-gamma producing Th1 cells (Trinchieri G, 1994; Macatonia SE, 1995). IL-10 is a pleiotropic cytokine, which promotes Th2 cell development (Stumbles PA, 1998; Iwasaki A, 1999). In contrast, IL-10-producing DCs may promote the generation of T regulatory cells with an immunosuppressive function (Wakkach A, 2003; Mocellin S, 2004). IL-10 has also been shown to inhibit the production of IL-12 by Th1 cells (Yang X, 1999; Moore KW, 2001).

In the present study, both wildtype lactobacilli and recombinant lactobacilli could induce BMDC maturation by upregulating the surface expression of CD80, CD86, CD40 and MHC class II I-A^b on BMDC (Figure 3.7) as well as the production of IL-12, IL-10, IL-6 and TNF-α (Figure 3.8). The surface marker expression profiles and cytokine production were found relatively similar among the same *Lactobacillus* strains, suggesting that the genetic transformation of *Lactobacillus plantarum* NC8 and LGG does not modify their intrinsic adjuvanicity in the DC immunomodulation. Interestingly, both wildtype LGG- and recombinant LGG-pulsed BMDCs significantly induced higher levels of IL-10 and IL-12 production as compared to that of wildtype *Lactobacillus* plantarum NC8- and recombinant Lactobacillus *plantarum* NC8-pulsed BMDCs (Figure 3.8A and 3.8B). Similar profiles with lower levels of cytokine production are observed in Flt3-derived DCs pulsed with either wildtype or recombinant *Lactobacillus* strain (Figure 3.9). Taken together, these results suggested that the *Lactobacillus* strains could modulate the cytokine production by murine DCs in a strain-dependent manner.

Akbari O et al. has reported that the pulmonary dendritic cells (DCs) from mice exposed to respiratory antigen transiently produced IL-10. These IL-10-producing DCs were phenotypically mature and migrate to the draining lymph nodes to stimulate the antigen-specific Tregs (Akbari O, 2001). In addition, mice lacking IL-10 exhibited spontaneous enterocolitis and exaggerated asthmatic and allergic

responses (Kuhn R, 1993). Based on these published data, the findings derived from this study denoted the possibility of recombinant LGG expressing Blo t 5 in the induction of IL-10-producing tolerogenic DCs which eventually drive to the antigen-specific Treg cell development. However, the immunological consequences of the relatively high IL-10 production by LGG-pulsed DCs remains to be further elucidated.

On the other hand, *Lactobacillus plantarum* was previously reported as a potent inducer of IL-12, directing the Th1 cell development (Murosaki S, 1998; Hessle C, 1999; Repa A, 2003). It might have the capacity to switch the established Th2 response in mite allergic patients towards Th1 response by inducing the production of IL-10 and IL-12 by monocyte-derived DCs from allergic patients (Pochard P, 2005). However, it appeared that wildtype and recombinant *Lactobacillus plantarum* NC8 tested in this study induced lower levels of IL-10 and IL-12 production by pulsed-DCs as compared to that of the LGG strain. The discrepancy in the findings between this study and the published data could be due to the differential adjuvant properties exhibited by the different sub-strains of *Lactobacillus plantarum*. For example, the levels of IL-12p70 produced by murine splenocytes from ovalbumin (OVA)-sensitised mice stimulated with OVA in the presence of *Lactobacillus plantarum* JCM 1149 and *Lactobacillus plantarum* MEP170401 were 39 and 4,907 pg/ml, respectively (Sashihara T, 2006). These

results suggested that different *Lactobacillus* substrains may possess their own intrinsic adjuvant properties.

Moreover, all wildtype and recombinant lactobacilli tested induced the production of TNF-α and IL-6 by the pulsed-BMDCs. The activation NF-κB via Toll-like receptors on the surface of DCs leads to the production of TNF-α proinflammatory cytokine (Medzhitov R, 2001). On the other hand, IL-6 is mainly produced by APCs. It has been reported that IL-6 promotes terminal differentiation of B cells into plasma cells, induces Th2 cell polarisation (Rincón M, 1997), and enhances the intestinal IgA response (McGhee JR, 1991; Ramsay AJ, 1994; Braciak TA, 2000). However, unlike IL-10 and IL-12, the precise roles of these two cytokines in the innate immunity are relatively unknown, thus the possible immunological impacts of IL-6 and TNF-α produced by lactobacilli-pulsed DCs remains to be elucidated.

Interestingly, the data on the T cell activation indicated that Blo t 5-expressing recombinant lactobacilli pulsed-BMDCs induced significantly higher levels of T cell proliferation and cytokine production as compared to that of the BMDCs pulsed with exogenously added Blo t 5 protein alone or in the combination of wildtype lactobacilli. Immature DCs uptake antigen by three ways: phagocytosis, macropinocytosis or adsorptive pinocytosis (Banchereau J, 1998). Immature DCs can uptake particles and microbes via phagocytosis (Inaba K, 1993; Svensson M,

1997). They can also form large pinocytic vesicles in which extracellular fluid and solutes are sampled, where a process called macropinocytosis (Sallusto F, 1995). They can also express receptors that mediate adsorptive endocytosis (Sallusto F, 1994; Sallusto F, 1995; Jiang W, 1995). At any rate, bacterium-associated antigen was presented with higher efficiency than soluble antigen by B cells, indicating that phagocytosis represents a very effective mechanism for providing antigens to the MHC class II processing pathway in different APC types (Vidard L, 1996; Inaba K, 1998; Corinti S, 1999). Therefore, it appeared that the pulsed-BMDCs could be more efficient in uptaking the Blo t 5-expressing recombinant lactobacilli and subsequently present Blo t 5 to the Blo t 5-specific T cells as compared to the BMDCs pulsed with exogeneous Blo t 5 protein alone. Notably, recombinant *Lactobacillus plantarum* NC8-pulsed BMDCs have led to higher specific T cell proliferation and cytokine production by these specific T cells as compared to that of the recombinant LGG-pulsed BMDCs. These results reflected that an antigen threshold is required for the activation of T cells. The differential magnitude of T cell activation could be due to the differential amount of Blo t 5 expressed in these two recombinant lactobacilli.

To our knowledge, this was the first study which described the *in vitro* immunological characterisation of two recombinant *Lactobacillus* strains with distinct adjuvant properties. With the use of pSIP412 expression vector, the results clearly demonstrated that high levels of Blo t 5 were expressed in the intracellular

compartment of in *Lactobacillus plantarum* NC8 and LGG than other expression systems. The *in vitro* data on DC-lactobacilli co-cultures revealed that *Lactobacillus plantarum* NC8 and LGG possess some differential modulatory effects on murine DCs, implying that these two *Lactobacillus* strains may exhibit significant differential intrinsic adjuvant properties *in vivo*. The *in vivo* immunomodulatory effects of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG using murine models will be further discussed in the next chapter.

Chapter 4: The *in vivo* evaluation of the recombinant lactobacilli in mouse allergy models

4.1 Introduction

The prevalence of house dust mite (HDM)-associated allergic diseases has alarmingly increased worldwide. HDM-associated allergic diseases are Th2-mediated inflammatory diseases that are well characterised by an imbalance of T helper-like immune responses with an exaggerated production of Th2 cytokines such as Interleukin (IL)-4, IL-5 and IL-13 leading to the production of allergen-specific immunoglobulin (Ig)-E. The pathological roles of allergen-specific Th2 cells and Th2 cytokines in the allergic reactions have been documented in humans and mice (Lloyd CM, 2001; Galli SJ, 2008). Briefly, IL-4 and IL-13 induce IgE class switching. IL-4 promotes Th2 cell differentiation whereas IL-5 is crucial for the activation and recruitment of eosinophils. IL-13 is a critical Th2 cytokine which mediates cellular responses relevant to asthma (Walter DM, 2001; Kuperman DA, 2002; Wills-Karps M, 2004). Current available treatments for allergy and asthma are mainly based on the pharmacological interventions such as treatment with antihistamines, glucocorticoids or β-agonists. However, the pharmacotherapeutic drugs are largely for symptomatic treatment of allergic diseases and patients suffering from chronic allergic diseases are required to take these drugs for life. The side effects of the long-term usage of these drugs

are the main health concerns. For example, glucocorticoids can result in decreased bone density in atopic children. New strategies, which provide specific and long-lasting protective effects by targeting and modulationg allergen- specific Th2 immune responses, are therefore highly desirable for the prevention and treatment of allergic diseases.

Dietary lactic acid bacteria (LAB) with GRAS status have been reported to exert beneficial health effects (Fuller R, 1989; Isolauri E, 2001; Mercenier A, 2003). The epidemiological studies have reported a difference in the gut microflora composition of allergic and healthy children, suggesting the possible role of *Lactobacillus* in the development of allergic diseases (Sepp E, 1997; Bjorksten B, 1999; Bottcher MF, 2000). Recent experimental data indicates that some *Lactobacillus* strains inhibit antigen-specific IgE production, modulate T cell responses, as well as suppress the allergen-induced airway inflammation and airway hyper-reactivity (Murosaki S, 1998; Shida K, 1998; Kruisselbrink A, 2001; Forsythe P, 2007; Feleszko W, 2007; Hisbergues M, 2007). In addition, some clinical trials have highlighted the anti-allergic effects of some *Lactobacillus* strains (Kalliomaki M, 2001; Kalliomaki M, 2003; Rosenfeldt V, 2003; Rautava S, 2005; Abrahamsson TR, 2007; Kukkonen K, 2007). To date, advanced genetic engineering has allowed the use of *Lactobacillus* for the development of an antigen-based oral vaccine. Few reports have demonstrated that recombinant lactobacilli are capable of modulating the allergen-specific immune responses

(Kruisselbrink A, 2001; Daniel C, 2006; Charng YC, 2006; Rigaux P, 2009). Taken together, allergen-expressing recombinant lactobacilli offer a promising and safer mode of prevention and treatment for allergic diseases.

As described in the chapter 3 of this thesis, recombinant *Lactobacillus plantarum* NC8 and recombinant LGG expressing Blo t 5 have been generated and evaluated by *in vitro* immunological studies. Protein quantification has revealed that high levels of Blo t 5 were expressed in the intracellular compartment of *Lactobacillus plantarum* NC8 and LGG. In addition, the *in vitro* data on DC-lactobacilli co-cultures have shown that *Lactobacillus plantarum* NC8 and LGG possess some differential modulatory effects on murine DCs, implying that these two *Lactobacillus* strains may exhibit differential intrinsic adjuvant properties *in vivo*. Therefore, it is hypothesised that these Blo t 5-expressing recombinant lactobacilli would probably be able to modulate DCs and lead to the induction of antigen-specific T cell responses that antagonise specific Th2 cell responses and allergic airway inflammation. The present study aims to study the immunogenicity and immunomodulatory effects of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG expressing Blo t 5 in mouse allergy models. Firstly, the capacity of recombinant *Lactobacillus plantarum* NC8 and recombinant LGG to prime Blo t 5-specific immune responses in naïve mice is determined. Secondly, their prophylactic and therapeutic anti-allergic and anti-inflammatory effects are evaluated in murine allergy models specifically induced by the Blo t 5 protein.

4.2 Results

4.2.1 The immunogenicity of recombinant lactobacilli *in vivo*

To investigate whether recombinant lactobacilli expressing Blo t 5 protein could prime both antibody and T cell responses against Blo t 5 *in vivo*, groups of four mice were orally fed with 10^{10} live recombinant LGG or recombinant *Lactobacillus plantarum* NC8 for 3 consecutive days for 3 weeks (Figure 4.1). PBS- and wildtype lactobacilli-fed mice were included as controls. Sera were collected weekly for antibody measurement by ELISA. Cells from spleen and mesenteric lymph nodes (MLN) were harvested for *in vitro* cell cultures. Culture supernatants were collected for the measurement of IL-5, IL-10, IFN-γ and TGF-β by ELISA.

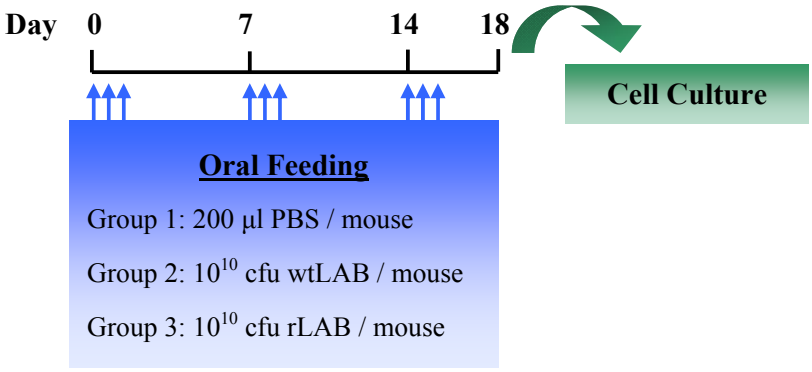


Figure 4.1 The experimental protocol I for the evaluation of *in vivo* immunogenicity of the Blo t 5 expressed in recombinant lactobacilli. Female C57BL/6 mice (n = 4) were orally fed with 200 µl of phosphate buffered saline (PBS) or 10^{10} colony formation units (cfu) of wildtype lactobacilli (wtLAB) or recombinant lactobacilli (rLAB) for three consecutive days per week over three weeks. The mice were sacrificed two days after the last feeding. Cells from mesenteric lymph nodes and spleens were harvested for *in vitro* cell cultures. Sera were collected by retro-orbital bleeding weekly for the measurement of Blo t 5-specific immunoglobulins.

4.2.1.1 The immunogenicity of recombinant *Lactobacillus plantarum* NC8

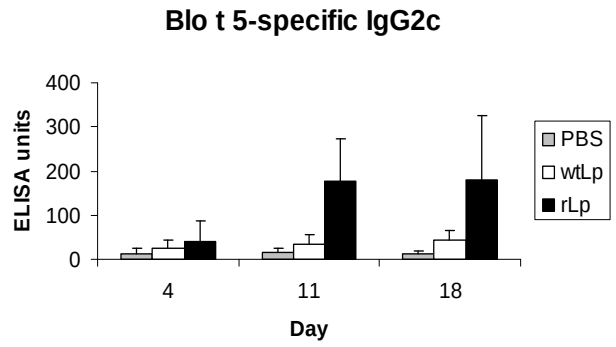
Mice fed with recombinant *Lactobacillus plantarum* NC8 showed higher levels of Blo t 5-specific IgG1 and IgG2c at day 11 or earlier up to day 18 (Figure 4.2). The level of Blo t 5-specific IgE in these mice were undetectable (data not shown). There was no detectable level of Blo t 5-specific antibody in wildtype *Lactobacillus plantarum* NC8- and PBS-fed mice.

Interestingly, only Blo t 5-stimulated MLN cells from mice fed with recombinant *Lactobacillus plantarum* NC8 produced a significant level of TGF-β (Figure 4.3A). The levels of IFN-γ in the culture supernatants were low in recombinant *Lactobacillus plantarum* NC8-fed mice and undetectable in wildtype *Lactobacillus plantarum*-fed mice as compared to that of the PBS-fed mice (Figure 4.3B). The levels of IL-5 and IL-10 were undetectable for all groups of mice.

Upon Blo t 5 stimulation, splenocytes from mice fed with recombinant *Lactobacillus plantarum* NC8 produced higher levels of IFN-γ and IL-10 as compared to that of the wildtype *Lactobacillus plantarum* NC8-fed mice (Figure 4.4B and 4.4C). The level of IL-10 was significantly higher in the splenic cultures of recombinant *Lactobacillus plantarum* NC8-fed mice than PBS-fed mice (Figure 4.4B). The levels of IL-5 production by the splenocytes were low in recombinant *Lactobacillus plantarum* NC8-fed mice and undetectable in the

control groups (Figure 4.4A). Wildtype *Lactobacillus plantarum* NC8-fed mice showed higher level of IFN-γ and significant increase in the level of IL-10 as compared to the PBS-fed mice (Figure 4.4B and 4.4C). Notably, the level of TGF-β was significantly lower in the splenic cultures of wildtype *Lactobacillus plantarum* NC8-fed mice as compared to that of the recombinant *Lactobacillus plantarum* NC8-fed mice (Figure 4.4D).

(A)



(B)

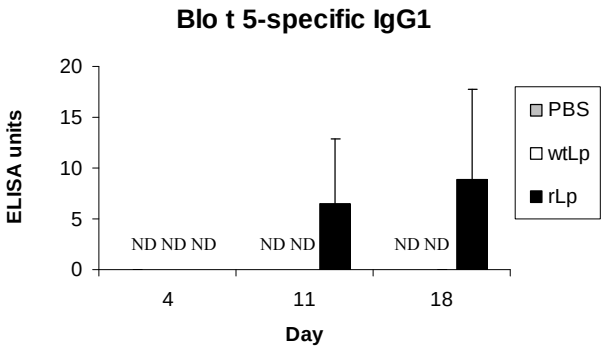


Figure 4.2 Oral feeding of recombinant *Lactobacillus plantarum* NC8 induced the production of Blo t 5-specific immunoglobulins in mice. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) as described in the Figure 4.1. The levels of Blo t 5-specific IgG2c (A) and IgG1 (B) were determined by enzyme-linked immunosorbent assays (ELISA). The antibody levels were expressed as arbitrary ELISA units. Data are presented as mean ± standard error of mean. ND: non-detectable.

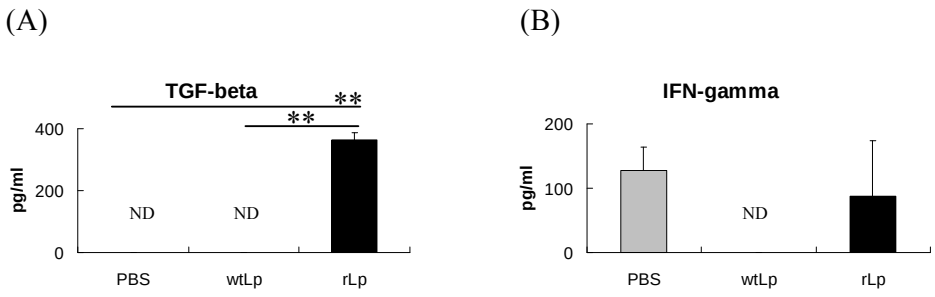


Figure 4.3 Oral feeding of recombinant *Lactobacillus plantarum* NC8 enhanced the production of TGF-β in mesenteric lymph node cultures. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) as described in the Figure 4.1. On day 18, cells from mesenteric lymph nodes were harvested and cultured with 10 μg/ml of Blo t 5 in the presence of antigen presenting cells. Culture supernatants were collected on day 5 for the measurement of TGF-β (A) and IFN-γ (B) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. **: $p < 0.01$; ND: non-detectable.

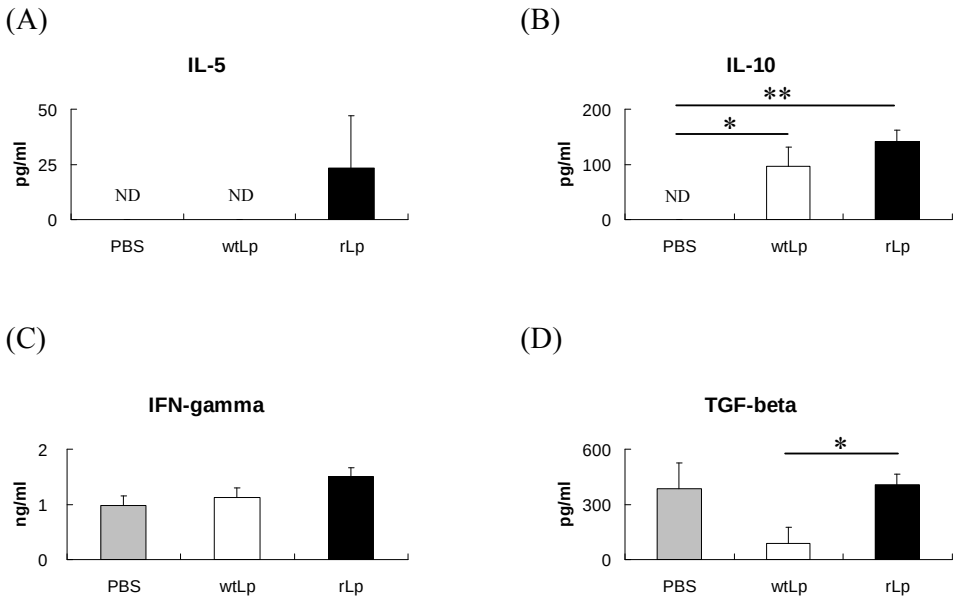


Figure 4.4 Oral feeding of recombinant *Lactobacillus plantarum* NC8 enhanced the production of cytokines in splenic cultures. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) as described in the Figure 4.1. On day 18, splenic cells were harvested and cultured with 10 μg/ml of Blo t 5. Culture supernatants were collected on day 5 for the measurement of IL-5 (A), IL-10 (B), IFN-γ (C) and TGF-β (D) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$; ND: non-detectable.

4.2.1.2 The immunogenicity of recombinant LGG

Naïve mice fed with recombinant LGG produced Blo t 5-specific IgG2c at day 18 (Figure 4.5). The levels of Blo t 5-specific IgE and IgG1 were undetectable in sera from all groups of mice (data not shown).

Upon Blo t 5 stimulation, only MLN cells from mice fed with the recombinant LGG produced a significant level of TGF-β (Figure 4.6A). Recombinant LGG-fed mice induced higher IFN-γ production as compared to that of the control groups (Figure 4.6B). The levels of IL-5 and IL-10 were undetectable in MLN cell cultures for all groups of mice (data not shown).

In the Blo t 5-stimulated splenic cultures, splenocytes from mice fed with recombinant LGG produced higher levels of IL-10, IFN-γ and TGF-β as compared to that of the control groups (Figure 4.7B, 4.7C and 4.7D). The levels of IL-5 production by the splenocytes were low in recombinant LGG-fed mice and undetectable in the control groups (Figure 4.7A).

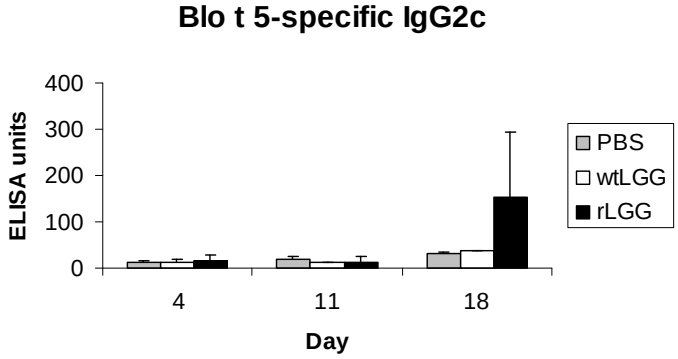


Figure 4.5 Oral feeding of recombinant *Lactobacillus rhamnosus* GG induced the Blo t 5-specific IgG2c production in mice. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) as described in the Figure 4.1. The levels of Blo t 5-specific IgG2c was determined by the enzyme-linked immunosorbent assay (ELISA) and expressed as arbitrary ELISA units. Data are presented as mean ± standard error of mean.

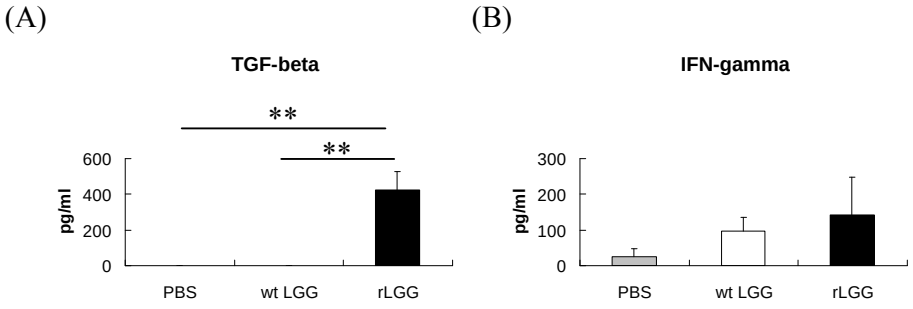


Figure 4.6 Oral feeding of recombinant *Lactobacillus rhamnosus* GG enhanced the production of IFN-γ and TGF-β in mesenteric lymph node cultures. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) as described in the Figure 4.1. On day 18, cells from mesenteric lymph nodes were harvested and cultured with 10 μg/ml of Blo t 5 in the presence of antigen presenting cells. Culture supernatants were collected on day 5 for the measurement of TGF-β (A) and IFN-γ (B) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. **: $p < 0.01$; ND: non-detectable.

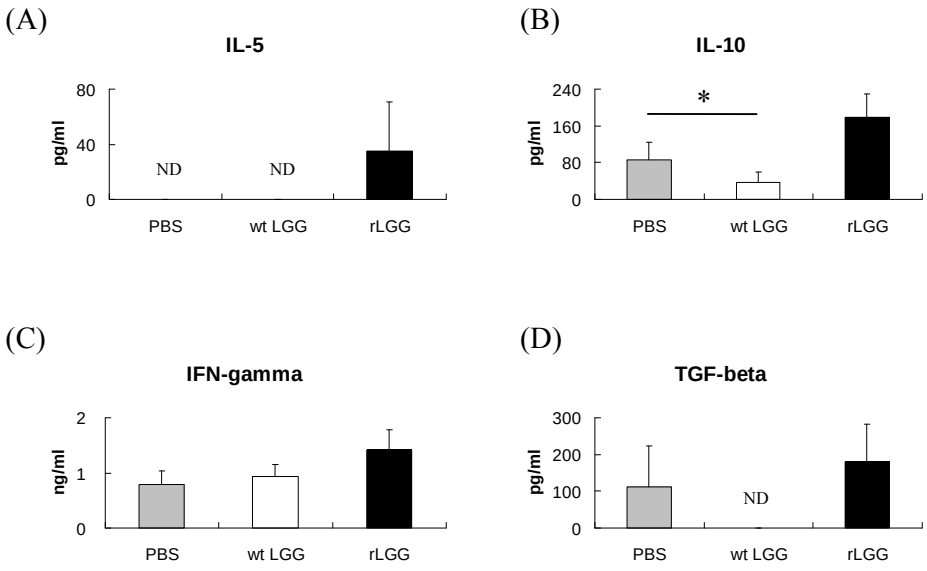


Figure 4.7 Oral feeding of recombinant *Lactobacillus rhamnosus* GG enhanced the production of cytokines in splenic cultures. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) as described in the Figure 4.1. On day 18, splenic cells were harvested and cultured with 10 μg/ml of Blo t 5. Culture supernatants were collected on day 5 for the measurement of IL-5 (A), IL-10 (B), IFN-γ (C) and TGF-β (D) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; ND: non-detectable.

4.2.2 The prophylactic anti-allergy effects of recombinant lactobacilli in a mouse allergy model

The potential application of the recombinant *Lactobacillus plantarum* NC8 and recombinant LGG as oral vaccines for the prevention of allergic diseases was evaluated *in vivo*. The experimental approach is schematically shown in Figure 4.8. Briefly, groups of four mice were orally fed with 10^{10} live recombinant lactobacilli for 3 consecutive days for the first 3 weeks. PBS- and wildtype lactobacilli-fed mice were served as controls. All mice were subsequently challenged by receiving intraperitoneal injections of Blo t 5 protein in alum at days 32 and 53, respectively. Sera were collected weekly for antibody measurement by ELISA. Two weeks after the last injection, all mice were sacrificed and cells from the MLN and spleens were harvested for *in vitro* cell cultures. Culture supernatants were collected for the measurement of IL-4, IL-5, IL-10, IL-13 and IFN-γ by ELISA.

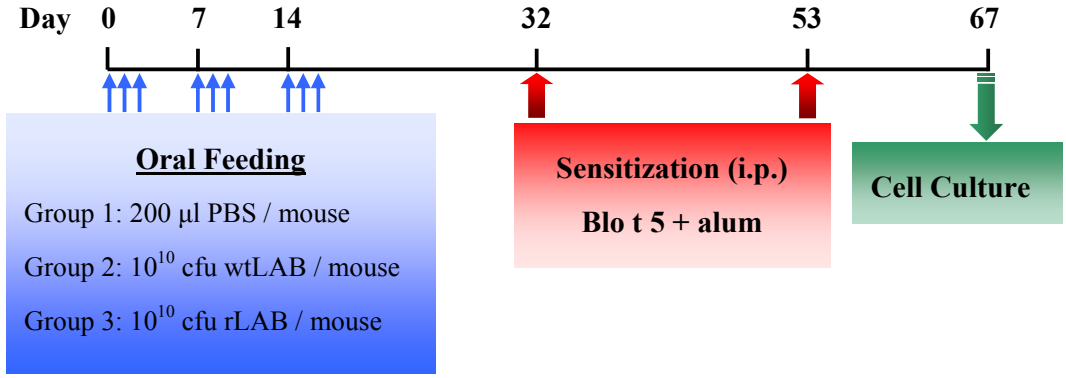


Figure 4.8 The experimental protocol II for the study of prophylactic effects of recombinant lactobacilli in the allergic murine model. Female C57BL/6 mice (n = 4) were orally fed with 200 µl of phosphate buffered saline (PBS), 10^{10} colony formation units (cfu) of wildtype lactobacilli (wtLAB) or recombinant lactobacilli (rLAB) for three consecutive days per week over three weeks. Sixteen days after the last feeding, all mice were intraperitoneally (i.p.) injected with 10 µg of Blo t 5 in 200 µl of PBS containing 4 mg of alum. Three weeks later, mice were received the second intraperitoneal injection of 5 µg of Blo t 5 in 200 µl of PBS containing 4 mg of alum. All mice were sacrificed on day 67. Cells from mesenteric lymph nodes and spleen were harvested for *in vitro* cell cultures. Sera were collected by retro-orbital bleeding weekly for the measurement of Blo t 5-specific immunoglobulins.

4.2.2.1 The prophylactic effects of recombinant *Lactobacillus plantarum* NC8

The Blo t 5-specific antibody production profiles in mice fed with recombinant *Lactobacillus plantarum* NC8 were shown in Figure 4.9. Notably, a week after the first sensitisation challenge of Blo t 5 protein, recombinant *Lactobacillus plantarum* NC8-fed mice showed a remarkable high-level production of Blo t 5-specific IgG2c, the signature IgG subclass for the Th1 responses. These mice also produced some Blo t 5-specific IgG1, but only basal levels of Blo t 5-specific IgE were detected. Conversely, mice fed with PBS or wildtype LAB produced higher levels of Blo t 5- specific IgE, but low levels of specific IgG2c, upon the Blo t 5 sensitisation challenge.

Upon Blo t 5 stimulation, MLN cells from recombinant *Lactobacillus plantarum* NC8-fed mice produced no detectable level of IL-4 (Figure 4.10A), lower levels of IL-10 (Figure 4.10B) as well as Th1 cytokine such as IFN-γ (Figure 4.10D) as compared to that of the control mice fed with PBS- or wildtype. Similar levels of IL-13 production were detected in MLN culture supernatants for all three groups (Figure 4.10C). Unlike mice fed with recombinant *Lactobacillus plantarum* NC8, mice fed with PBS or wildtype LAB showed no protection against Blo t 5 sensitisation challenge as reflected by the increased production of Th2 cytokines such as IL-4, IL-10 and IL-13 by the Blo t 5-stimulated MLN cells from these

mice (Figure 4.10). There was no detectable level of IL-5 produced by MLN cells from all three groups.

Blo t 5-stimulated splenocytes from recombinant *Lactobacillus plantarum* NC8-fed mice produced significant levels of IL-13, IL-10 and IFN-γ (Figure 4.11C, 11.1D and 4.11E), but no undetectable levels of IL-4 and IL-5 (Figure 4.11A and 4.11B). On the other hand, Blo t 5-stimulated splenocytes from mice fed with PBS or wildtype LAB produced the Th2 signature cytokines such as IL-4, IL-5 and IL-13. Higher levels of IL-10 and IFN-γ were produced by the stimulated splenocytes from wildtype LAB-fed mice as compared to that of the PBS-fed mice.

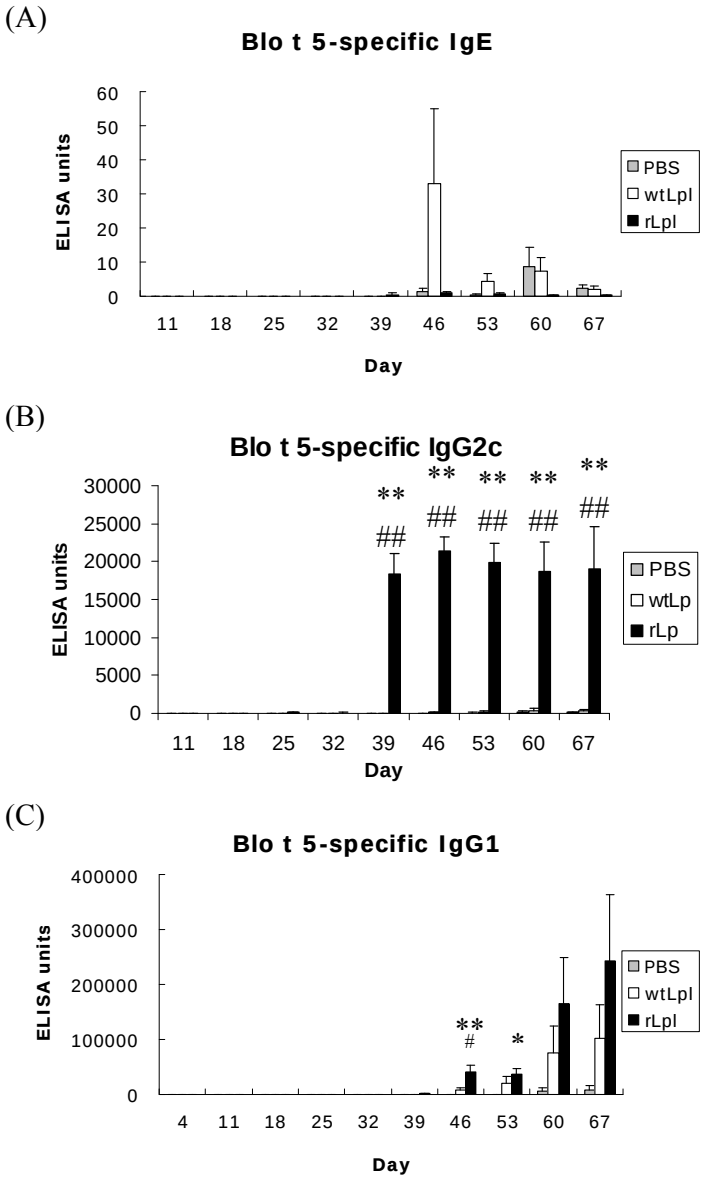


Figure 4.9 Oral feeding of recombinant *Lactobacillus plantarum* NC8 suppressed the production of Blo t 5-specific IgE and induced the production of Blo t 5-specific IgG1 and IgG2c in mice. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) followed by the challenge of Blo t 5 with alum. Sera were collected weekly for the antibody measurement. The levels of Blo t 5-specific IgE (A), IgG2c (B) and IgG1 (C) were measured by enzyme-linked immunosorbent assays (ELISA) and expressed as arbitrary ELISA units. Data are presented as mean ± standard error of mean. *: $p < 0.05$ as compared to the PBS-fed group; **: $p < 0.01$ as compared to the PBS-fed group; #: $p < 0.05$ as compared to the wildtype *Lactobacillus plantarum* NC8-fed group; ##: $p < 0.01$ as compared to the wildtype *Lactobacillus plantarum* NC8-fed group.

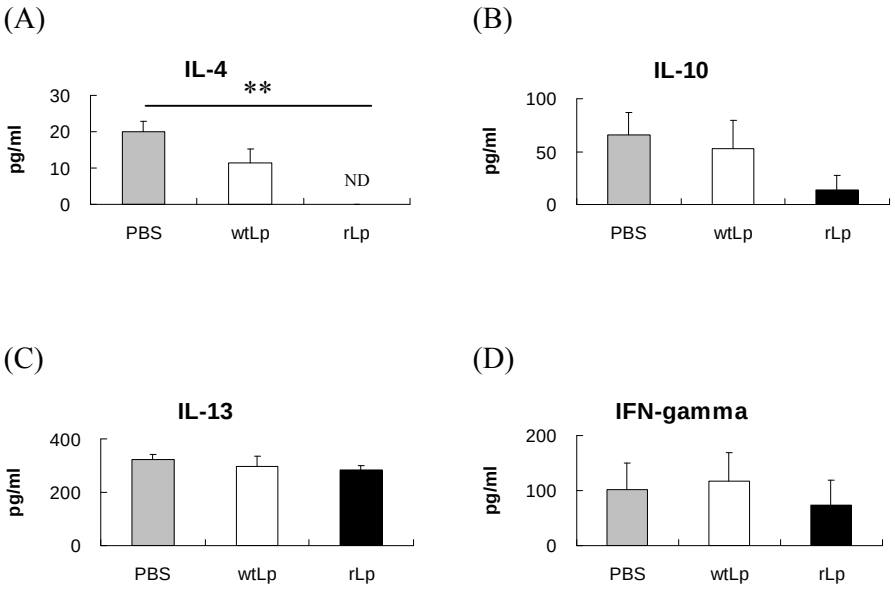


Figure 4.10 The cytokine profile of mesenteric lymph node cultures from mice fed with recombinant *Lactobacillus plantarum* NC8. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) followed by the challenge of Blo t 5 with alum. Two weeks later, cells from mesenteric lymph nodes were harvested and cultured with 10 μg/ml of Blo t 5 in the presence of antigen presenting cells. Culture supernatants were collected on day 3 for the measurement of IL-4 (A), IL-10 (B), IL-13 (C) and IFN-γ (D) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. **: $p < 0.01$; ND: non-detectable.

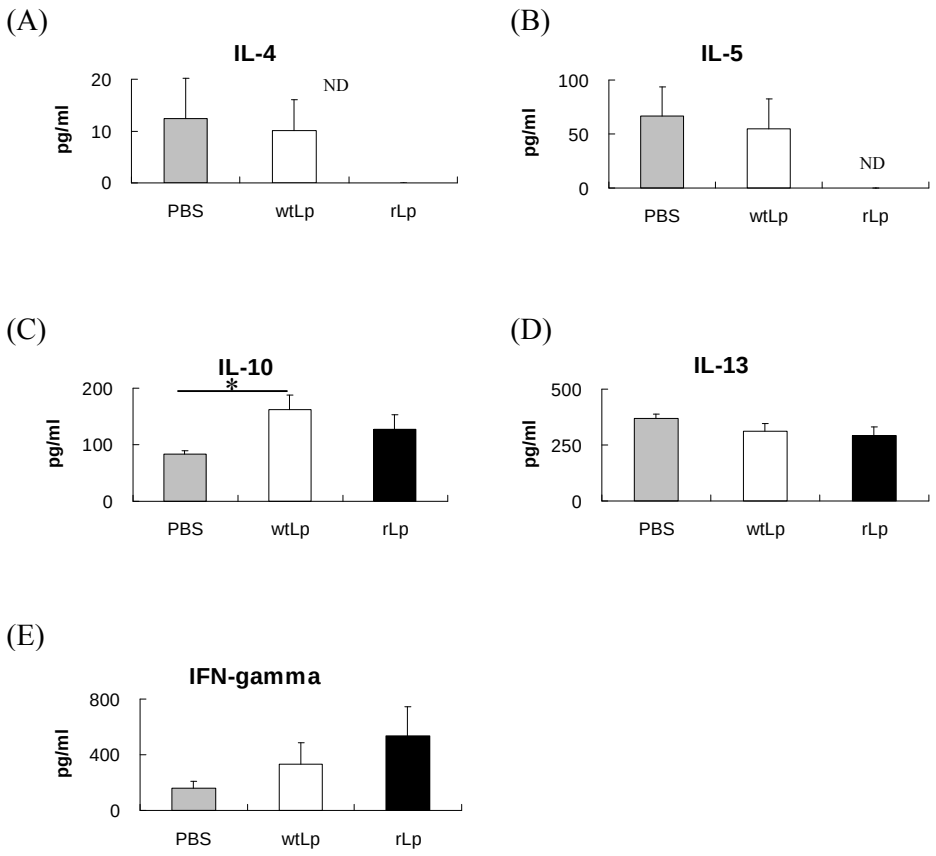


Figure 4.11 The cytokine profile of splenocyte cultures from mice fed with recombinant *Lactobacillus plantarum* NC8. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus plantarum* NC8 (wtLp, white bar) or recombinant *Lactobacillus plantarum* NC8 (rLp, black bar) followed by the challenge of Blo t 5 with alum. Two weeks later, splenocytes were collected and cultured with 10 μg/ml of Blo t 5. Culture supernatants were collected on day 3 for the measurement of IL-4 (A), IL-5 (B), IL-10 (C), IL-13 (D) and IFN-γ (E) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; ND: non-detectable.

4.2.2.2 The prophylactic effects of recombinant LGG

The Blo t 5-specific antibody production profiles in mice fed with the recombinant LGG-fed mice was shown in Figure 4.12. Differential levels of Blo t 5-specific IgE were detected in sera from all three groups of mice, but the levels of the Blo t 5-specific IgE in recombinant LGG-fed mice were significantly lower than that in the control mice. Recombinant LGG-fed mice also produced higher levels of Blo t 5-specific IgG2c and IgG1 than the control mice after the intraperitoneal challenge with Blo t 5 protein in alum.

Interestingly, when the MLN cells from three groups of mice were stimulated with Blo t 5 protein, a significant reduction in IL-13 level was detected in the MLN cultures of the recombinant LGG-fed mice than that of the PBS- and wildtype LGG-fed mice (Figure 4.13). The levels of IL-4, IL-5, IL-10 and IFN-γ were undetectable for all groups.

Blo t 5-stimulated splenocytes from recombinant LGG-fed mice also showed a significantly lower level of IL-13 production as compared to that of the PBS-fed mice (Figure 4.14B). The levels of IL-10 and IFN-γ produced by the splenic cultures of recombinant LGG-fed mice were found lower than that of the control mice (Figure 4.14A and 4.14C). Interestingly, as compared to the splenocytes from PBS-fed mice, Blo t 5-stimulated splenic cultures of wildtype LGG-fed mice

produced higher levels of IL-10 and IFN-γ and a significantly reduced level of IL-13 (Figure 4.14B).

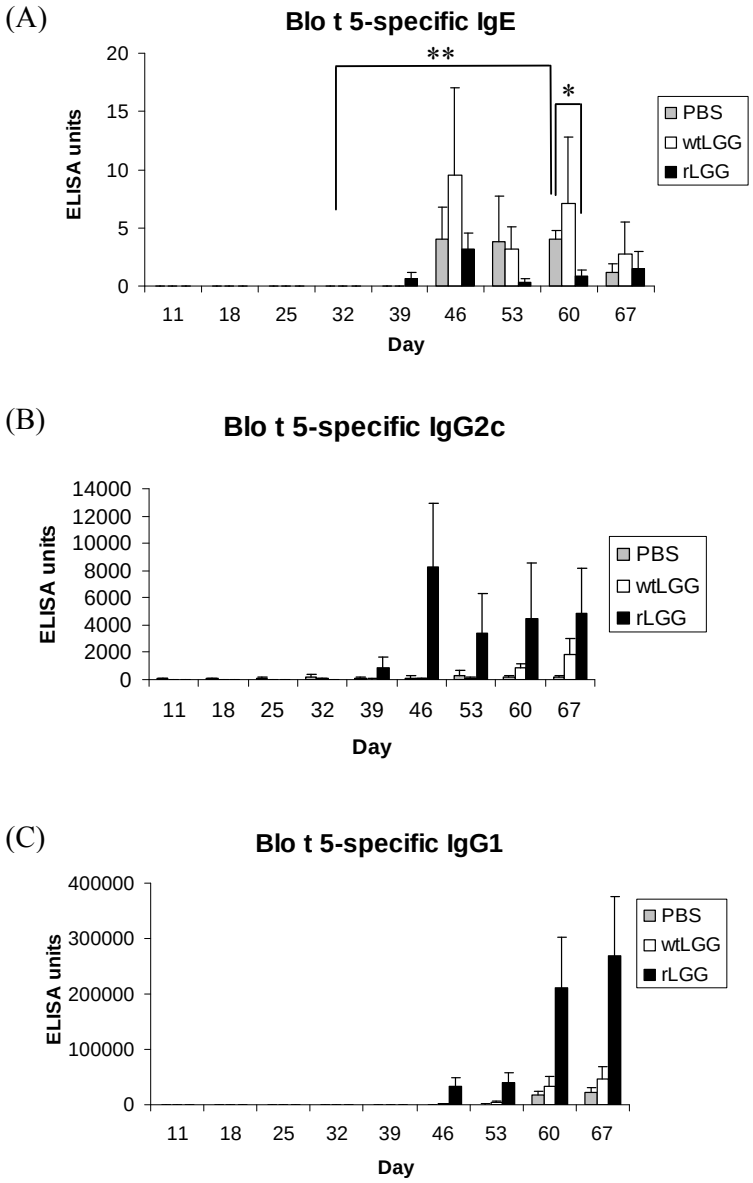


Figure 4.12 Oral feeding of recombinant *Lactobacillus rhamnosus* GG suppressed the production of Blo t 5-specific IgE and induced the production of Blo t 5-specific IgG1 and IgG2c in mice. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar) or wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) followed by the challenge of Blo t 5 with alum. Sera were collected weekly for the antibody measurement by enzyme-linked immunosorbent assays (ELISA). The levels of Blo t 5-specific IgE (A), IgG2c (B) and IgG1 (C) were measured and expressed as arbitrary ELISA units. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$.

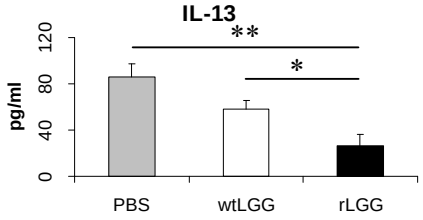


Figure 4.13 Oral feeding of recombinant *Lactobacillus rhamnosus* GG suppressed the IL-13 production in mesenteric lymph node cultures. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) followed by the challenge of Blo t 5 with alum. Two weeks later, cells from mesenteric lymph nodes were harvested and cultured with 10 μg/ml of Blo t 5 in the presence of antigen presenting cells. Culture supernatants were collected on day 3 for the IL-13 measurement by the enzyme-linked immunosorbent assay. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$.

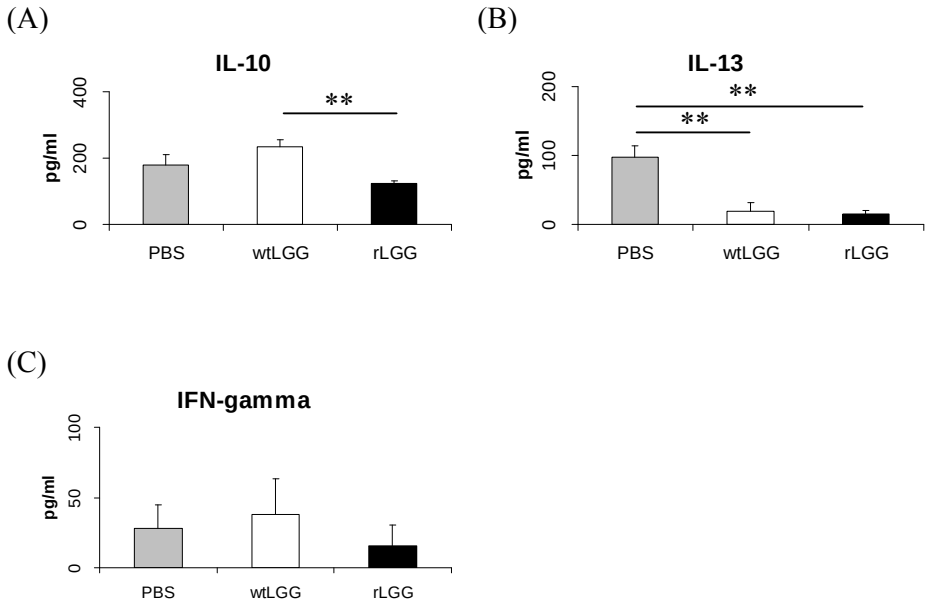


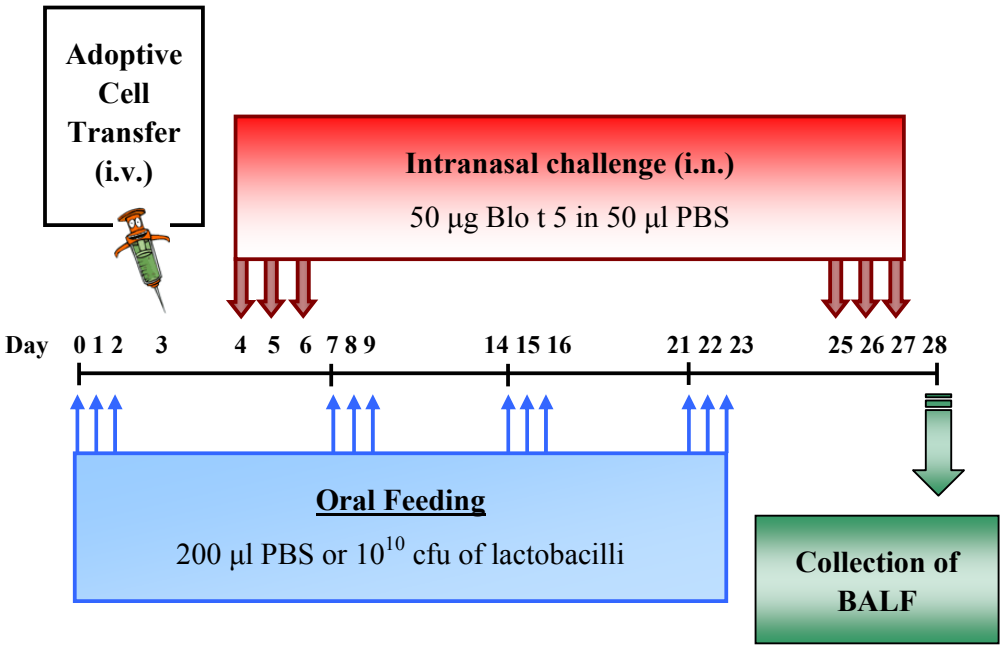
Figure 4.14 The cytokine profile of splenocyte cultures from mice fed with recombinant *Lactobacillus rhamnosus* GG. Mice were immunized as described in the Figure 4.8. Mice were orally fed with phosphate buffered saline (PBS, grey bar), wildtype *Lactobacillus rhamnosus* GG (wtLGG, white bar) or recombinant *Lactobacillus rhamnosus* GG (rLGG, black bar) followed by the challenge of Blo t 5 with alum. Two weeks later, splenocytes were collected and cultured with 10 μg/ml of Blo t 5. Culture supernatants were collected on day 3 for the measurement of IL-10 (A), IL-13 (B) and IFN-γ (C) by enzyme-linked immunosorbent assays. Data are presented as mean ± standard error of mean. *: $p < 0.05$; **: $p < 0.01$.

4.2.3 The evaluation of the anti-inflammatory effects of recombinant lactobacilli in an allergic airway inflammation mouse model

To determine whether the recombinant *Lactobacillus plantarum* NC8 and recombinant LGG could attenuate the allergen-induced allergic airway inflammation, mice sensitised by Blo t 5-specific Th2 cells were fed with two recombinant *Lactobacillus* strains. The protocol is schematically shown in Figure 4.15. Briefly, groups of four mice were orally fed with 10^{10} live recombinant lactobacilli for 3 consecutive days throughout the duration of the experiment. PBS- and wildtype lactobacilli-fed mice served as controls. Mice were adoptively transferred with PBS or Blo t 5-specific Th2 cells via tail vein injection at day 3. All mice were intranasally challenged with Blo t 5 at day 4, 5, 6, 25, 26 and 27. At day 28, mice were sacrificed and bronchoalveolar lavage fluids (BALF) were collected for the analysis of infiltrating cells by differential cell count.

The cell infiltrates in BALF were examined by the cytospin slide preparations stained with Liu stain. As compared to the mice adoptively transferred with Blo t 5-specific T cells, PBS-fed mice which were adoptively transferred with PBS (PBS/PBS), showed lower total cell number in BALF (Figure 4.16A). The infiltrating cell types in BALF of PBS/PBS mice were predominantly macrophages and monocytes (Figure 4.16B). As compared to PBS/PBS group, decreased numbers of macrophages and monocytes and increased numbers of eosinophils and neutrophils were observed in PBS-fed mice sensitised by the

adoptively transferred Blo t 5-specific T cells (PBS/Bt5 cells). These data indicated that these mice (PBS/Bt5 cells) developed allergic airway inflammation upon the intranasal challenge of Blo t 5 protein. Notably, lower percentage of eosinophils and higher percentage of macrophages and monocytes were found in the airway of the recombinant LGG-fed mice (rLGG/Bt5 cells) after intranasal challenge with Blo t 5 protein as compared to the control group (PBS/Bt5 cells). Conversely, the percentage of eosinophils was higher in wildtype LGG-fed mice (wtLGG/Bt5 cells) as compared to the control group (PBS/Bt5 cells). The percentages of cell types in wildtype *Lactobacillus plantarum* NC8 (wtLp/Bt5 cells) and recombinant *Lactobacillus plantarum* NC8 (rLp/Bt5 cells) were found similar to that of the control group (PBS/Bt5 cells). These findings suggested that recombinant LGG could confer some degree of protection against Th2 sensitisation, resulting in the attenuation of the airway inflammation in recombinant LGG-fed mice.



Group	Oral Feeding	i.v.	i.n.
Group 1:	PBS	PBS	Blo t 5
Group 2:	PBS	Th2 cells	Blo t 5
Group 3:	wildtype *Lactobacillus plantarum* NC8	Th2 cells	Blo t 5
Group 4:	recombinant *Lactobacillus plantarum* NC8	Th2 cells	Blo t 5
Group 5:	wildtype *Lactobacillus rhamnosus* GG	Th2 cells	Blo t 5
Group 6:	recombinant *Lactobacillus rhamnosus* GG	Th2 cells	Blo t 5

Figure 4.15 The experimental protocol III for the study of protective effects of recombinant lactobacilli in an allergic airway inflammation model. Female C57BL/6 mice (n = 4) were received 200µl of phosphate buffered saline (PBS), 10^{10} colony formation units cfu wildtype lactobacilli or 10^{10} cfu recombinant lactobacilli for three consecutive days per week for four weeks. Both recombinant *Lactobacillus plantarum* NC8 and recombinant *Lactobacillus rhamnosus* GG strains were tested in this protocol. On day 3, a total of 2×10^6 of Blo t 5-specific Th2 cells in 150 µl of PBS was intravenously (i.v.) transferred into mice via the tail vein. Mice were intranasally (i.n.) challenged with 50 µg of Blo t 5 in 50 µl PBS for the next three consecutive days after the cell transfer. All mice were received intranasal challenge of Blo t 5 for three consecutive days from day 25 onwards. Twenty-four hours later, mice were sacrificed. Bronchoalveolar lavage fluids (BALF) were collected for differential cell counts.

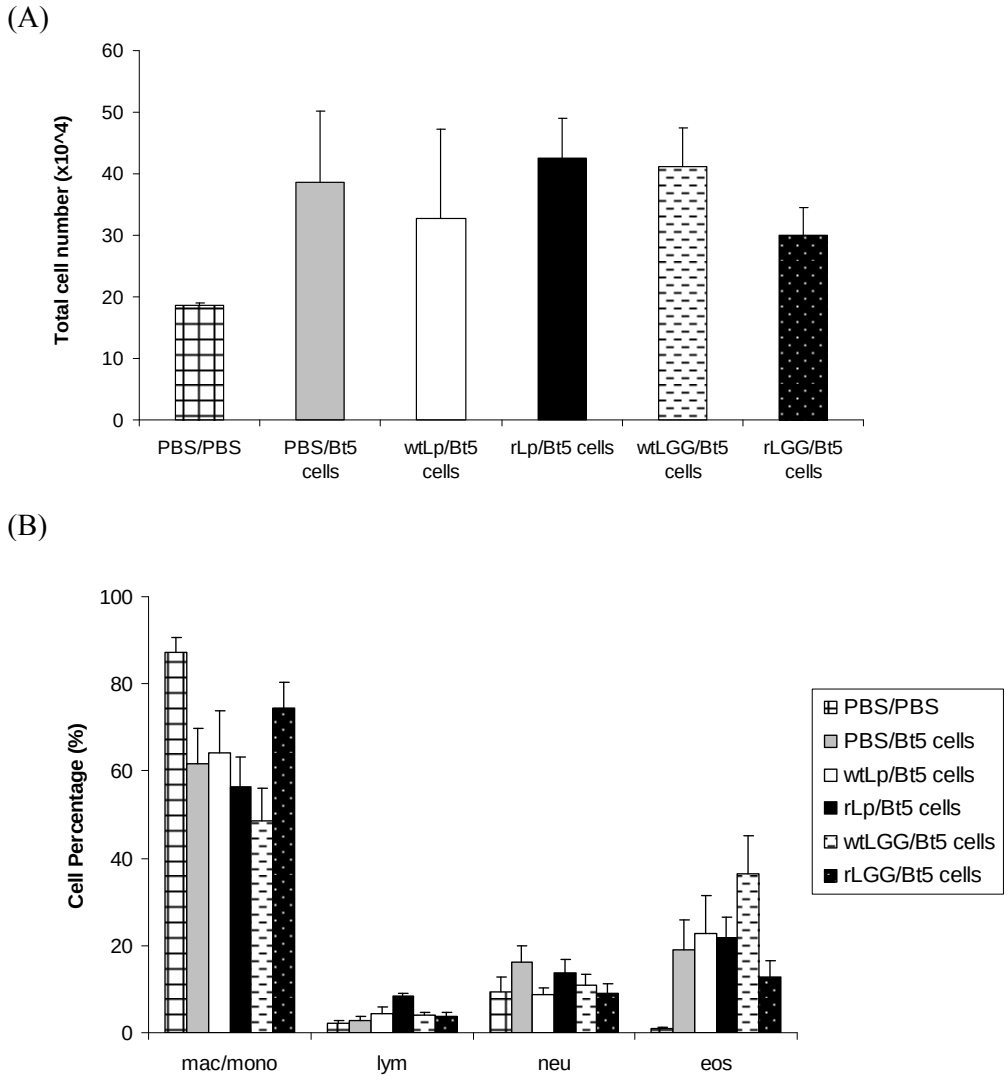


Figure 4.16 Oral feeding of recombinant *Lactobacillus rhamnosus* GG but not recombinant *Lactobacillus plantarum* NC8 reduced the allergic airway inflammation in lungs. Mice were immunized as described in the Figure 4.15. The total numbers of cellular infiltrates (A) and differential cell counts (B) in the bronchoalveolar lavage fluids of phosphate buffered saline (PBS, grey bar)-, wildtype *Lactobacillus plantarum* NC8 (white bar)-, recombinant *Lactobacillus plantarum* NC8 (black bar)-, wildtype *Lactobacillus rhamnosus* GG (horizontally hatched bar)- and recombinant *Lactobacillus rhamnosus* GG (dotted bar)-fed mice were shown. The percentages of macrophages/monocytes (mac/mono), lymphocytes (lym), neutrophils (neu) and eosinophils (eos) were derived from the number of each cell type divided with 500 counted cells examined at the 1000-fold magnification. Data are represented as mean ± standard error of mean.

4.3 Discussions

In the present study, the immunogenicity of both recombinant *Lactobacillus plantarum* NC8 and recombinant LGG was first evaluated by feeding naive mice with these Blo t 5-expressing recombinant LAB. Blo t 5-specific B and T cell responses in these mice were examined by immunological analysis. These results showed that both strains of recombinant lactobacilli induced Blo t 5-specific antibodies. The levels of specific antibodies were detectable in sera from recombinant *Lactobacillus plantarum* NC8-fed mice as early as day four, whereas the production of specific antibody in recombinant LGG-fed mice was only detectable at day 18. As aforementioned, the amount of Blo t 5 expressed by recombinant LGG was about 2.5-fold lower than that of the recombinant *Lactobacillus plantarum* NC8 (Table 3.1). The differential kinetics of antibody responses might be attributable to the differential levels of Blo t 5 expressed in these recombinant lactobacilli. Notably, both strains of recombinant lactobacilli induced high levels of specific IgG2c, with relatively low levels of specific IgG1 and no detectable level of specific IgE. These findings may suggest that the recombinant LAB preferentially primed Th1-skewed immunity in these mice.

MLNs are the crucial checkpoint for the priming and induction of antigen-specific T cells and oral tolerance. Studies using adoptively transferred TCR-transgenic T cells demonstrated that the antigen recognition occurs in the MLNs within few hours of feeding protein (Smith KM, 2002). MLNs are required for the induction

of oral tolerance (Spahn TW, 2002). TGF-β-producing T helper 3 (Th3) cells can be isolated by repeated restimulation of MLN or splenic lymphocytes from mice fed with low doses of antigen (Miller A, 1992). These regulatory T cells probably have an important role in the IgA class switching (Mowat AM, 2003). Th3 cells are normally triggered in an antigen-specific manner, but the suppressive effects can be exerted in an antigen-non-specific fashion. They can also exert "by-stander suppression" when they encounter the fed antigen in target organ (Weiner HL, 2001). In this study, the Blo t 5-stimulated MLN cells from recombinant lactobacilli-fed mice produced significantly higher levels of TGF-β with low levels of IFN-γ (Figure 4.3A&B and Figure 4.6A&B). These data suggested that recombinant lactobacilli-uploaded DCs could prime and induce TGF-β-producing antigen-specific Th3 cells in MLNs environment. Th3 cells are $Foxp3^+$ $CD4^+$ T cells that may play a role in T cell tolerance and suppression activity. Further studies are required to characterise the phenotype and function of these antigen-specific T cells found in MLNs.

Upon Blo t 5 stimulation, splenocytes from recombinant lactobacilli-fed mice produced low levels (slightly above basal levels) of IL-5, IFN-γ and TGF-β, but the IL-10 production was more significant as compared to the controls. On the basis of the cytokine profiles, there was no distinctly clear subset of polarised T cells observed. However, the elevated IL-10 production by the splenocytes may suggest a possible induction of T regulatory cell 1 (Tr1) cells characterised by

production of IL-10. Tr1 cells are $CD4^+$ $Foxp3^-$ IL-10-producing adaptive Treg cells. It has been suggested that the mechanism of the therapeutic effect of allergen-specific immunotherapy for allergy involved the induction of adaptive Treg cells, such as IL-10 secreting Tr1 cells (Bohle B, 2007; Meiler F, 2008).

Taken together, the cytokine data derived from the Blo t 5-stimulated MLN and splenocytes cultures revealed that both recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were capable of inducing Blo t 5-specific T and B cell responses in the treated mice. In addition, the data also suggested that these recombinant lactobacilli may have the capability to prime the induction of Blo t 5-specific Th3 and Tr1 in these mice. However, further studies are required to confirm these preliminary data.

Next, the prophylactic anti-allergy effects of both recombinant *Lactobacillus plantarum* NC8 and recombinant LGG were examined in a murine allergy model. In this study, recombinant lactobacilli, wildtype lactobacilli or PBS-fed mice were challenged with Blo t 5 sensitisation by two intraperitoneal injections with Blo t 5 protein in alum. The production of Blo t 5-specific antibodies in mice sera was examined by ELISA. The ELISA data revealed that all mice fed with recombinant lactobacilli showed attenuated production of Blo t 5-specific IgE accompanied by high-level production of Blo t 5-specific IgG2c. It is conceivable that the production of Blo t 5-specific IgG2c in these mice was first primed by the Blo t

5-expressing recombinant lactobacilli and then greatly boosted by the intraperitoneal injections of Blo t 5 protein in alum. Blo t 5-specific IgG2c was about 2.5-fold lower in recombinant LGG-fed mice as compared to recombinant *Lactobacillus plantarum* NC8-fed mice. Again, this may be due to the fact that recombinant LGG produced lower amount of Blo t 5 protein resulting in a weaker priming of Blo t 5-specific immune responses. Since IgG2c is the signature antibody for Th1-skewed responses, the high levels of IgG2c is indicative of the strong Th1-skewed Blo t 5-specific T cell responses that mediated the suppression of Th2-mediated production of Blo t 5-specific IgE. The production of Blo t 5-specific IgG1 which was remarkably elevated after the second injection of Blo t 5 protein in alum was observed in these mice. Further studies are needed to elucidate the precise underlying reason for the differential antibody production profiles and their immunological implications.

At the cellular level, Blo t 5-stimulated cells of MLNs and spleens from recombinant *Lactobacillus plantarum* NC8-fed mice showed suppression of IL-4 and IL-5 but not IL-13 production as compared to that of the control mice (Figures 4.10 and 4.11). Higher production of IFN-γ and IL-10 was found in the splenic cultures of recombinant *Lactobacillus plantarum* NC8-fed mice as compared to that of the PBS-fed mice. Taken together, the antibody and T cytokine production profiles may infer that recombinant *Lactobacillus plantarum* NC8 could downregulate the Th2-skewed allergic responses by the immunoregulatory

suppression mediated by both IL-10 and IFN-γ produced by the T regulatory and Th1 cells induced by the recombinant *Lactobacillus plantarum* NC8.

On the other hand, similar experiments conducted with recombinant and wildtype LGG yielded distinctively different cytokine production profiles by MLN cells and splenocytes from the treated mice. The IL-13 levels in both MLN and splenic cultures of recombinant LGG-fed mice were significantly attenuated as compared to the PBS-fed controls. In addition, the production of IL-10 and IFN-γ by the Blo t 5 stimulated splenocytes from recombinant LGG-fed mice was not upregulated. It appears that there was a general suppression of cytokine production by Blo t 5-specific T cells. Unlike the scenario seen in mice treated with recombinant *Lactobacillus plantarum* NC8, there was no evidence to support the possible induction of antigen-specific T-regulatory or Th1 cells in recombinant LGG-fed mice. Therefore, the underlying mechanism in the suppression of Th2 responses by the recombinant LGG is yet to be elucidated.

Experimental asthma mouse model established using Blo t 5-specific Th2 cells was used to determine whether recombinant *Lactobacillus plantarum* NC8 and recombinant LGG could suppress the allergen-induced allergic airway inflammation. The results showed that only mice fed with recombinant LGG showed attenuated airway inflammation upon the intranasal challenge with Blo t 5 in these sensitised mice as indicated by the reduction of eosinophils infiltrating

into the allergen-challenged airways (Figure 4.16). It is worth noting that Blo t 5-stimulated MLN and spleen cells from mice fed with recombinant LGG showed undetectable IL-4 and IL-5 as well as remarkable attenuation of IL-13 production (Figures 4.13 and 4.14B). This is an interesting observation in view of the fact that all these Th2 effector cytokines play crucial roles in allergic inflammation. IL-5 enhances recruitment and survival of eosinophils in the airway. IL-13 is a potent effector cytokine that induces the central features of asthma, such as eosinophilia, mucus production, airway hyper-reactivity (Wills-Karp M, 1998; Kuperman DA, 2002). The attenuation of IL-13 may be one of the mechanisms for the reduction of airway inflammation.

Another possible mechanism underlying the anti-allergic inflammation could be linked to the possibility of recombinant LGG expressing Blo t 5 induced IL-10-producing tolerogenic DCs which eventually drive to the antigen-specific Treg cell development as discussed in chapter 3. It has been reported that genetically engineered DCs expressing IL-10 could induce antigen-specific tolerance in OVA-induced experimental model for airway allergy, probably through the action of OVA-specific $CD4^{+}CD25^{+}Foxp3^{+}$ IL-10-producing regulatory T cells (Henry E, 2008). The precise mechanisms for the anti-allergic inflammation in the airway induced by the recombinant LGG should be a major focus of the future studies.

In summary, these *in vivo* studies suggested that both the recombinant *Lactobacillus plantarum* NC8 and recombinant LGG, but not their wildtype counterparts, are potentially useful for the prevention of allergen sensitisation. However, it appears that recombinant LGG is more effective than recombinant *Lactobacillus plantarum* NC8 in the attenuation of established allergic airway inflammation

Chapter 5

Conclusion and Future Prospects

Allergic asthma is one of the most important lung inflammatory diseases worldwide and sensitisation by *Dermatophagoides pteronyssinus* (Der p) and *Blomia tropicalis* (Blo t) allergens is the main trigger for the development of allergic asthma. To date, allergen-specific immunotherapy (SIT) represents the only aetiology treatment for allergic diseases, by providing an allergen-specific and long term protective immune response. The concept of using recombinant lactobacilli expressing allergens as oral vaccines for the prevention and treatment of allergic diseases has been explored in recent years. Several published results were promising but preliminary, thus further studies are necessary to dissect the underlying mechanisms for the optimisation of vaccine efficacy for clinical applications.

Among others, two key factors such as the choice of *Lactobacillus* strain and the amount of antigen produced by the recombinant *Lactobacillus* strains are crucial in the design of a live vector for efficient oral vaccination. *Lactobacillus rhamnosus* GG is one of the best-studied probiotic bacteria for anti-allergy whereas *Lactobacillus plantarum* NC8 is a common *Lactobacillus* strain for high-level antigen expression. In this study, both *Lactobacillus* strains were

chosen as the host strains for the expression of Blo t 5 major mite allergen using an inducible pSIP412 expression system. The results showed that both recombinant lactobacilli expressed high levels of Blo t 5 in their intracellular compartment. As dendritic cell (DC)-derived cytokines play the most important role in the T cell polarization towards Th1 or Th2 or Treg cell development, the immunomodulatory effects of the two recombinant lactobacilli on murine DCs were therefore examined by *in vitro* studies. Notably, both recombinant lactobacilli exhibited differential modulatory effects on the cytokine production by DCs. Recombinant *Lactobacillus rhamnosus* GG-pulsed DCs significantly induced higher levels of IL-10 and IL-12 production as compared to that of the recombinant *Lactobacillus plantarum* NC8-pulsed DCs. Both recombinant lactobacilli-pulsed dendritic cells effectively uptake recombinant lactobacilli, processed and presented Blo t 5 to Blo t 5-specific T cells and subsequently led to the activation of a Blo t 5-specific T cell line. These *in vitro* findings implied that recombinant lactobacilli possess their own distinct adjuvanicity, induce differential immunomodulatory effects on cytokine production by DCs and potentially able to drive the differential polarisation of Th1, Th2 or T regulatory subsets.

The *in vivo* immunogenicity studies demonstrated that both recombinant *Lactobacillus plantarum* NC8 and recombinant *Lactobacillus rhamnosus* GG were capable of inducing the Blo t 5-specific T cell responses and Blo t 5-specific

Th1-skewed IgG2c without any detectable Blo t 5-specific IgE priming. This study suggested the potential use of these recombinant lactobacilli for the oral priming of a protective anti-allergy immunity. Therefore, their respective prophylactic effects were further investigated in an allergy murine model. Interestingly, the findings clearly showed that both recombinant *Lactobacillus* strains but not wildtype *Lactobacillus* strains could downregulate the production of Blo t 5-specific IgE and upregulate the production of Blo t 5-specific IgG2c in Blo t 5-sensitised mice. Moreover, the oral administration of recombinant *Lactobacillus* strains led to the downregulation of Th2 cytokine production by mesenteric lymph node cells and splenocytes. Strikingly, a reduction of the eosinophils infiltration into BALF was shown in recombinant *Lactobacillus rhamnosus* GG-fed mice. These anti-inflammatory effects were not observed in recombinant *Lactobacillus plantarum* NC8-fed mice. Taken together, these findings suggested that both recombinant *Lactobacillus* strains could be potential promising candidates for the development of preventive oral vaccine for mite-allergy triggered allergic diseases. However, the recombinant *Lactobacillus rhamnosus* GG instead of the recombinant *Lactobacillus plantarum* NC8, appears to be a better candidate for the development of therapeutic oral vaccine for treatment of allergic inflammation associated with allergic asthma. It is noteworthy that the use of pSIP412 expression system in *Lactobacillus rhamnosus* GG as a host strain for Blo t 5 expression and the evaluation of the recombinant *Lactobacillus rhamnosus* GG for the prevention and treatment of allergic asthma

have not been reported. This study is probably the first of such studies in the field. However, the precise underlying mechanisms of the immunomodulatory effects of these recombinant lactobacilli remain to be further elucidated.

Several aspects in this study require further extensive investigation. The optimisation in the immunisation protocol represents one of the key areas to be addressed. The optimised dosage of both recombinant lactobacilli required to prime protective anti-allergic immune responses by antigen-specific immunomodulation, has to be determined by further dose response *in vivo* studies. Moreover, the identification and characterisation of the T cell subsets are crucial for elucidating the underlying mechanisms that antagonise the Th2 immune responses. *In vitro* and *in vivo* studies targeting at the interactions of these recombinant lactobacilli with antigen presenting cells such as DCs, and other immune and non-immune cells in the gut environment are highly necessary to understand the various differential modes of action of these recombinant lactobacilli. In addition, the possible role of IL-10-producing DCs induced by recombinant *Lactobacillus rhamnosus* GG in the suppression of Th2 immune responses and airway inflammation needs further in-depth investigations by a combination of cellular and molecular approaches involving techniques such as flow cytometric analysis, real-time quantitative RT-PCR, adoptive cell transfer experiments and so forth. Studies using genetically modified mice such as IL-10-deficient mice, will be also useful in addressing the mechanistic questions.

Further information derived from all these studies will facilitate the development of *Lactobacillus*-based oral vaccine for allergy and allergic diseases.

REFERENCES

Abrahamsson TR, Jakobsson T, Böttcher MF, Fredrikson M, Jenmalm MC, Björkstén B, Oldaeus G. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol. 2007. 119:1174-80.

Adel-Patient K, Ah-Leung S, Creminon C, Nouaille S, Chatel JM, Langella P, Wal JM. Oral administration of recombinant Lactococcus lactis expressing bovine beta-lactoglobulin partially prevents mice from sensitization. Clin Exp Allergy. 2005. 35:539-46.

Ahrne S, Nobaek S, Jeppsson B, Adlerberth I, Wold AE, Molin G. The normal Lactobacillus flora of healthy human rectal and oral mucosa. J Appl Microbiol. 1998. 85:88-94.

Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol. 2001. 2:725-31.

Albert ML, Jegathesan M, Darnell RB. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. Nat Immunol. 2001. 2:1010-7.

Arlian LG, Bernstein D, Bernstein IL, Friedman S, Grant A, Lieberman P, Lopez M, Metzger J, Platts-Mills T, Schatz M. Prevalence of dust mites in the homes of people with asthma living in eight different geographic areas of the United States. J Allergy Clin Immunol. 1992. 90:292-300.

Arlian LG, Bernstein IL, Vyszenski-Moher DL, Gallagher JS. Antigenicity and allergenicity of body and fecal extracts of the mite Dermatophagoides pteronyssinus (Acari: Pyroglyphidae). J Med Entomol. 1987. 24:252-9.

Arlian LG, Morgan MS, Neal JS. Dust mite allergens: ecology and distribution. Curr Allergy Asthma Rep. 2002. 2:401-11.

Arlian LG. Dust mites: update on their allergens and control. Curr Allergy Asthma Rep. 2001. 1:581-6.

Arruda LK, Fernandez-Caldas E, Naspitz CK, Montealegre F, Vailes LD, Chapman MD. Identification of Blomia tropicalis allergen Blo t 5 by cDNA cloning. Int Arch Allergy Immunol. 1995. 107:456-7.

Arshad SH, Bateman B, Matthews SM. Primary prevention of asthma and atopy during childhood by allergen avoidance in infancy: a randomised controlled study. Thorax. 2003. 58:489-93.

Arshad SH. Allergen avoidance and prevention of atopy. Curr Opin Allergy Clin Immunol. 2004. 4:119-23.

Aukrust T, Blom H. transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res Int. 1992. 25:253-261.

Aukrust TW, Brurberg MB, Nes IF. Transformation of *Lactobacillus* by electroporation. Methods Mol Biol. 1995. 47:201-8.

Axelsson L, Lindstad G, Naterstad K. Development of an inducible gene expression system for Lactobacillus sakei. Lett Appl Microbiol. 2003. 37:115-20.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol. 2000. 18:767-811.

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998. 392:245-52.

Beijerinck MW. Sur les ferments lactiques de l'industrie. Archives Néerlandaises des Sciences Exactes et Naturelles. 1901. 6:212–243.

Bermúdez-Humarán LG, Cortes-Perez NG, Le Loir Y, Alcocer-González JM, Tamez-Guerra RS, de Oca-Luna RM, Langella P. An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. J Med Microbiol. 2004. 53:427-33.

Björkstén B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy. 1999. 29:342-6. Erratum in: Clin Exp Allergy. 2000. 30:1047.

Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol. 2001. 108:516-20.

Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol. 2007. 120:707-13.

Böttcher MF, Nordin EK, Sandin A, Midtvedt T, Björkstén B. Microflora-associated characteristics in faeces from allergic and nonallergic infants. Clin Exp Allergy. 2000. 30:1590-6.

Braciak TA, Gallichan WS, Graham FL, Richards CD, Ramsay AJ, Rosenthal KL, Gauldie J. Recombinant adenovirus vectors expressing interleukin-5 and -6 specifically enhance mucosal immunoglobulin A responses in the lung. Immunology. 2000. 101:388-96.

Brouwer ML, Wolt-Plompen SA, Dubois AE, van der Heide S, Jansen DF, Hoijer MA, Kauffman HF, Duiverman EJ. No effects of probiotics on atopic dermatitis in infancy: a randomized placebo-controlled trial. Clin Exp Allergy. 2006. 36:899-906.

Bussmann C, Maintz L, Hart J, Allam JP, Vrtala S, Chen KW, Bieber T, Thomas WR, Valenta R, Zuberbier T, Sager A, Novak N. Clinical improvement and immunological changes in atopic dermatitis patients undergoing subcutaneous immunotherapy with a house dust mite allergoid: a pilot study. Clin Exp Allergy. 2007. 37:1277-85.

Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med. 1996. 184:747-52.

Chew FT, Lim SH, Goh DY, Lee BW. Sensitization to local dust-mite fauna in Singapore. Allergy. 1999. 54:1150-9.

Chew FT, Yi FC, Chua KY, Fernandez-Caldas E, Arruda LK, Chapman MD, Lee BW. Allergenic differences between the domestic mites Blomia tropicalis and Dermatophagoides pteronyssinus. Clin Exp Allergy. 1999. 29:982-8.

Chew FT, Zhang L, Ho TM, Lee BW. House dust mite fauna of tropical Singapore. Clin Exp Allergy. 1999. 29:201-6.

Choi CH, Kim TI, Lee SK, Yang KM, Kim WH. Effect of Lactobacillus GG and conditioned media on IL-1beta-induced IL-8 production in Caco-2 cells. Scand J Gastroenterol. 2008. 43:938-47.

Christensen HR, Frøkiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol. 2002. 168:171-8.

Chua KY, Cheong N, Kuo IC, Lee BW, Yi FC, Huang CH, Liew LN. The Blomia tropicalis allergens. Protein & Peptide Lett. 2007.14: 325-333.

Chua KY, Huangfu T, Liew LN. DNA vaccines and allergic diseases. Clin Exp Pharmacol Physiol. 2006. 33:546-50.

Coeuret V, Dubernet S, Bernardeau M, Guéguen M, Vernoux JP. Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. Lait. 2003. 83:269–306.

Collins MD, Rodrigues UM, Ash C, Aguirre M, Farrow JAE, Martinez-Mrurcia A, Phillips BA, Williams AM, Wallbanks S. Phylogenetics analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of the 16S rRNA. FEMS Microbiol Lett. 1991. 77:5-12.

Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. Nat Rev Immunol. 2008. 8:435-46.

Corinti S, Medaglini D, Cavani A, Rescigno M, Pozzi G, Ricciardi-Castagnoli P, Girolomoni G. Human dendritic cells very efficiently present a heterologous antigen expressed on the surface of recombinant gram-positive bacteria to CD4+ T lymphocytes. J Immunol. 1999. 163:3029-36.

Corry DB. IL-13 in allergy: home at last. Curr Opin Immunol. 1999. 11:610-4.

Cortes-Perez NG, Lefèvre F, Corthier G, Adel-Patient K, Langella P, Bermúdez-Humarán LG. Influence of the route of immunization and the nature of the bacterial vector on immunogenicity of mucosal vaccines based on lactic acid bacteria. Vaccine. 2007. 25:6581-8.

Daniel C, Repa A, Wild C, Pollak A, Pot B, Breiteneder H, Wiedermann U, Mercenier A. Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1. Allergy. 2006. 61:812-9.

de Ruyter PG, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM. Functional analysis of promoters in the nisin gene cluster of Lactococcus lactis. J Bacteriol. 1996. 178:3434-9.

de Ruyter PG, Kuipers OP, de Vos WM. Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl Environ Microbiol. 1996. 62:3662-7.

de Vos WM. Gene cloning and expression in lactic streptococci. FEMS Microbiol Rev. 1987. 46:281–295.

Dieye Y, Hoekman AJ, Clier F, Juillard V, Boot HJ, Piard JC. Ability of Lactococcus lactis to export viral capsid antigens: a crucial step for development of live vaccines. Appl Environ Microbiol. 2003. 69:7281-8.

Drakes M, Blanchard T, Czinn S. Bacterial probiotic modulation of dendritic cells. Infect Immun. 2004. 72:3299-309.

Eijsink VG, Axelsson L, Diep DB, Håvarstein LS, Holo H, Nes IF. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Van Leeuwenhoek. 2002. 81:639-54.

Fallon PG, Jolin HE, Smith P, Emson CL, Townsend MJ, Fallon R, Smith P, McKenzie AN. IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. Immunity. 2002. 17:7-17.

Fang H, Elina T, Heikki A, Seppo S. Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol. 2000. 29:47-52.

Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, Ahrens B, Groneberg DA, Wahn U, Hamelmann E. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy. 2007. 37:498-505.

Fernández-Caldas E, Fox RW, Bucholtz GA, Trudeau WL, Ledford DK, Lockey RF. House dust mite allergy in Florida. Mite survey in households of mite-sensitive individuals in Tampa, Florida. Allergy Proc. 1990. 11:263-7.

Fernández-Caldas E, Lockey RF. Blomia tropicalis, a mite whose time has come. Allergy. 2004. 59:1161-1164.

Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med. 2004. 10:475-80.

Fuller R, Perdigón G. Probiotics 3: Immunomodulation by the Gut Microflora and Probiotics. Springer. 2000.

Fuller R. Probiotics in man and animals. J Appl Bacteriol. 1989. 66:365-78.

Furrie E. Probiotics and allergy. Proc Nutr Soc. 2005. 64:465-9.

Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008. 454:445-54.

Garrido D, Suau A, Pochart P, Cruchet S, Gotteland M. Modulation of the fecal microbiota by the intake of a Lactobacillus johnsonii La1-containing product in human volunteers. FEMS Microbiol Lett. 2005. 248:249-56.

Gasson MJ. Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. J Bacteriol. 1983. 154:1-9.

Georas SN, Guo J, De Fanis U, Casolaro V. T-helper cell type-2 regulation in allergic disease. Eur Respir J. 2005. 26:1119-37.

Goh LT, Kuo IC, Luo S, Chua KY, White W. Production and purification of recombinant *Blomia tropicalis* group 5 allergen from *Pichia pastori*s culture. Biotechnology Letters 2001. 23:661-665.

Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, MCloskey N, Coker HA, Fear D, Smurthwaite L. The biology of IgE and the basis of allergic disease. Annu Rev Immunol. 2003. 21:579-628.

Grangette C, Müller-Alouf H, Geoffroy M, Goudercourt D, Turneer M, Mercenier A. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and in vivo persistence. Vaccine. 2002. 20:3304-9.

Grangette C, Müller-Alouf H, Goudercourt D, Geoffroy MC, Turneer M, Mercenier A. Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum. Infect Immun. 2001. 69:1547-53.

Grangette C, Müller-Alouf H, Hols P, Goudercourt D, Delcour J, Turneer M, Mercenier A. Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. Infect Immun. 2004. 72:2731-7.

Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med. 1996. 184:19-29.

Grünig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. Science. 1998. 282:2261-3.

Hammes WP, Vogel RF. The genus Lactobacillus. The Genera of Lactic Acid Bacteria. .Wood BJB, Holzapfel WH editions. Blackie Academic & Professional, Glasgow, UK. 1995. Vol 2:19-54.

Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ. Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut. 2004. 53:1602-9.

Havenaar R, Huis In't Veld JMJ. Probiotics: a general view. In: Lactic acid bacteria in health and disease. Elsevier Applied Science Publishers, 1992. Vol 1.

Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol. 2005. 5:271-83.

Helin T, Haahtela S, Haahtela T. No effect of oral treatment with an intestinal bacterial strain, Lactobacillus rhamnosus (ATCC 53103), on birch-pollen allergy: a placebo-controlled double-blind study. Allergy. 2002. 57:243-6.

Henry E, Desmet CJ, Garzé V, Fiévez L, Bedoret D, Heirman C, Faisca P, Jaspar FJ, Gosset P, Jacquet AP, Desmecht D, Thielemans K, Lekeux P, Moser M, Bureau F. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. J Immunol. 2008. 181:7230-42.

Hessle C, Hanson LA, Wold AE. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol. 1999. 116:276-82.

Heufler C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur J Immunol. 1996. 26:659-68.

Hilkens CM, Kalinski P, de Boer M, Kapsenberg ML. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood. 1997. 90:1920-6.

Hisbergues M, Magi M, Rigaux P, Steuve J, Garcia L, Goudercourt D, Pot B, Pestel J, Jacquet A. In vivo and in vitro immunomodulation of Der p 1 allergen-specific response by Lactobacillus plantarum bacteria. Clin Exp Allergy. 2007. 37:1286-95.

Ho PS, Kwang J, Lee YK. Intragastric administration of Lactobacillus casei expressing transmissible gastroentritis coronavirus spike glycoprotein induced specific antibody production. Vaccine. 2005. 23:1335-42.

Holgate ST. The epidemic of allergy and asthma. Nature. 1999. 402:B2-4.

Holo H, Nes IF. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp *cremoris* grown with glycine in osmotically stabilized media. Appl Environ Microbiol. 1989. 55:3119-3123.

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science. 1993. 260:547-9.

Hsu CH, Chua KY, Tao MH et al. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyper responsiveness in vivo by genetic immunization. Nat Med. 1996. 2: 540–4.

Iliev ID, Tohno M, Kurosaki D, Shimosato T, He F, Hosoda M, Saito T, Kitazawa H. Immunostimulatory oligodeoxynucleotide containing TTTCGTTT motif from Lactobacillus rhamnosus GG DNA potentially suppresses OVA-specific IgE production in mice. Scand J Immunol. 2008. 67:370-6.

Inaba K, Inaba M, Naito M, Steinman RM. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J Exp Med. 1993. 178:479-88.

Inaba K, Turley S, Yamaide F, Iyoda T, Mahnke K, Inaba M, Pack M, Subklewe M, Sauter B, Sheff D, Albert M, Bhardwaj N, Mellman I, Steinman RM. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J Exp Med. 1998. 188:2163-73.

Isolauri E, Arvola T, Sütas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy. 2000. 30:1604-10.

Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J Exp Med. 1999. 190:229-39.

Iwasaki A. Mucosal dendritic cells. Annu Rev Immunol. 2007. 25:381-418.

Jarman ER, Lamb JR. Reversal of established CD4+ type 2 T helper-mediated allergic airway inflammation and eosinophilia by therapeutic treatment with DNA vaccines limits progression towards chronic inflammation and remodelling. Immunology. 2004. 112:631-42.

Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature. 1995. 375:151-5.

Kajikawa A, Satoh E, Leer RJ, Yamamoto S, Igimi S. Intragastric immunization with recombinant Lactobacillus casei expressing flagellar antigen confers antibody-independent protective immunity against Salmonella enterica serovar Enteritidis. Vaccine. 2007. 25:3599-605.

Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G. Monomeric IgE Stimulates Signaling Pathways in Mast Cells that Lead to Cytokine Production and Cell Survival. Immunity. 2001. 14:801-11.

Kaliński P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today. 1999. 20:561-7.

Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol. 2001. 107:129-34.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 2001. 357:1076-9.

Kalliomäki M, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet. 2003. 361:1869-71.

Kandler O, Weiss N. The Genus: *Lactobacillus*. In: Sneath PHA., Mair NS, Sharpe ME, Holt JG. Bergey's Manual of Systematics Bacteriology. Williams and Wilkins. 1986. Vol 2:1209-1234.

Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol. 2003. 3:984-93.

Kay AB. 100 years of 'Allergy': can von Pirquet's word be rescued? Clin Exp Allergy. 2006.36:555-9.

Kay AB. Allergy and allergic diseases. First of two parts. N. Engl. J. Med. 2001. 344, 30–37.

Kelsall BL, Rescigno M. Mucosal dendritic cells in immunity and inflammation. Nat Immunol. 2004. 5:1091-5.

Kim N, Kwon SS, Lee J, Kim S, Yoo TJ. Protective effect of the DNA vaccine encoding the major house dust mite allergens on allergic inflammation in the murine model of house dust mite allergy. Clin Mol Allergy. 2006. 4:4.

Kips JC. Cytokines in asthma. Eur Respir J Suppl. 2001. 34: 24s-33s.

Kirjavainen PV, Salminen SJ, Isolauri E. Probiotic bacteria in the management of atopic disease: underscoring the importance of viability. J Pediatr Gastroenterol Nutr. 2003. 36:223-7.

Kleerebezem M, Beerthuyzen MM, Vaughan EE, de Vos WM, Kuipers OP. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for Lactococcus, Leuconostoc, and Lactobacillus spp. Appl Environ Microbiol. 1997. 63:4581-4.

Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kämpgen E, Romani N, Schuler G. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J Exp Med. 1996 Aug 1;184:741-6. Erratum in: J Exp Med. 1996. 184:following 1590.

Kronin V, Hochrein H, Shortman K, Kelso A. Regulation of T cell cytokine production by dendritic cells. Immunol Cell Biol. 2000. 78:214-23.

Krüger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delwar D, van Dalen PJ, Pouwels PH, Leer RJ, Kelly CG, van Dollenweerd C, Ma JK, Hammarström L. In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol. 2002. 20:702-6.

Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993. 75:263–74.

Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol. 2007. 119:192-8.

Kuo IC, Yi FC, Cheong N, Shek LP, Chew FT, Lee BW, Chua KY. Sensitization to Blomia tropicalis and dermatophagoides pteronyssinus-a comparative study between Singapore and Taiwan. Asian Pac J Allergy Immunol. 1999. 17:179-88.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002. 8:885-9.

Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol. 2006. 6:761-71.

Lau S, Illi S, Sommerfeld C, Niggemann B, Bergmann R, von Mutius E, Wahn U. Early exposure to house-dust mite and cat allergens and development of childhood asthma: a cohort study. Multicentre Allergy Study Group. Lancet. 2000. 356:1392-7.

Lee VHL, Dodda-Kashi S, Grass GM, Rubas W. Oral route of peptide and protein drug delivery. New York: Marcel Dekker. 1991. 691-738.

Leer RJ, van Luijk N, Posno M, Pouwels PH. Structural and functional analysis of two cryptic plasmids from Lactobacillus pentosus MD353 and Lactobacillus plantarum ATCC 8014. Mol Gen Genet. 1992. 234:265-74.

Liu CH. A new rapid method of staining thin blood film: first report. J Formosan Med Assoc. 1953. 52:348-352.

Lloyd CM, Gonzalo JA, Coyle AJ, Gutierrez-Ramos JC. Mouse models of allergic airway disease. Adv Immunol. 2001. 77:263-95.

Lutz MB, Kukutsch N, Ogilvie AL, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999. 223:77–92.

Maassen CB, Laman JD, den Bak-Glashouwer MJ, Tielen FJ, van Holten-Neelen JC, Hoogteijling L, Antonissen C, Leer RJ, Pouwels PH, Boersma WJ, Shaw DM. Instruments for oral disease-intervention strategies: recombinant Lactobacillus casei expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine. 1999. 17:2117-28.

Maassen CB, van Holten-Neelen C, Balk F, den Bak-Glashouwer MJ, Leer RJ, Laman JD, Boersma WJ, Claassen E. Strain-dependent induction of cytokine profiles in the gut by orally administered Lactobacillus strains. Vaccine. 2000. 18:2613-23.

Maassen CB. A rapid and safe plasmid isolation method for efficient engineering of recombinant lactobacilli expressing immunogenic or tolerogenic epitopes for oral administration. J Immunol Methods. 1999. 223:131-6.

Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol. 1995. 154:5071-9.

Macfarlane S, Furrie E, Cummings JH, Macfarlane GT. Chemotaxonomic analysis of bacterial populations colonizing the rectal mucosa in patients with ulcerative colitis Clin Infect Dis. 2004. 38:1690-1699.

Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immunol. 1997. 99:179-85.

Manolio TA, Barnes KC, Naidu RP, Levett PN, Beaty TH, Wilson AF. Correlates of sensitization to Blomia tropicalis and Dermatophagoides pteronyssinus in asthma in Barbados. Int Arch Allergy Immunol. 2003. 131:119-26.

Mariana A, Ho TM, Sofian-Azirun M, Wong AL. House dust mite fauna in the Klang Valley, Malaysia. Southeast Asian J Trop Med Public Health. 2000. 31:712-21.

Marks GB, Mihrshahi S, Kemp AS, Tovey ER, Webb K, Almqvist C, Ampon RD, Crisafulli D, Belousova EG, Mellis CM, Peat JK, Leeder SR. Prevention of asthma during the first 5 years of life: a randomized controlled trial. J Allergy Clin Immunol. 2006. 118:53-61.

Mathiesen G, Sorvig E, Blatny J, Naterstad K, Axelsson L, Eijsink VG. High-level gene expression in Lactobacillus plantarum using a pheromone-regulated bacteriocin promoter. Lett Appl Microbiol. 2004. 39:137-43.

Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. J Leukoc Biol. 2004. 75:721-30.

McGhee JR, Fujihashi K, Beagley KW, Kiyono H. Role of interleukin-6 in human and mouse mucosal IgA plasma cell responses. Immunol Res. 1991. 10:418-22.

Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001. 1:135-45.

Meiler F, Zumkehr J, Klunker S, Rückert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. J Exp Med. 2008. 205:2887-98.

Merk K, Borelli C, Korting HC. Lactobacilli bacteria-host interactions with special regard to the urogenital tract. Int J of Med Microbio. 2005. 295:9-18.

Metchnikoff E. The Prolongation of life. GP Putnam. 1910. 109-116.

Metchnikoff E. The prolongation of life. Optimistic studies. 1907.

Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. Proc Natl Acad Sci U S A. 1992. 89:421-5.

Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev. 2004. 15:61-76.

Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, Bavari S, Klaenhammer TR. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc Natl Acad Sci U S A. 2005. 102:2880-5.

Molin G, Jeppsson B, Johansson ML, Ahrne S, Nobaek S, Stahl M, Bengmark S. Numerical taxonomy of Lactobacillus spp. associated with healthy and diseased mucosa of the human intestines. J Appl Bacteriol. 1993. 74:314-323.

Montaner LJ, da Silva RP, Sun J, Sutterwala S, Hollinshead M, Vaux D, Gordon S. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest. 1999. 103:779-88.

Moog F. The lining of the small intestine. Sci Am. 1981. 245:154-8,160,162 et passiom.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001. 19:683-765.

Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996. 17:138-46.

Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol. 2003. 3:331-41.

Mudde GC, Hansel TT, von Reijsen FC, Osterhoff BF, Bruijnzeel-Koomen CA. IgE: an immunoglobulin specialized in antigen capture? Immunol Today. 1990. 11:440-3.

Murosaki S, Yamamoto Y, Ito K, Inokuchi T, Kusaka H, Ikeda H, Yoshikai Y. Heat-killed Lactobacillus plantarum L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. J Allergy Clin Immunol. 1998. 102:57-64.

Naik MT, Chang CF, Kuo IC, Kung CC, Yi FC, Chua KY, Huang TH. Roles of structure and structural dynamics in the antibody recognition of the allergen proteins: an NMR study on Blomia tropicalis major allergen. Structure. 2008. 16:125-36.

Nes IF, Eijsink VG. Regulation of group II peptide bacteriocins synthesis by quorum-sensing mechanisms. In Cell-Cell Signaling in Bacteria. G. M. Dunny & S.C. Winans editions. Washington, DC: American Society of Microbiology. 1999. 175-192.

Niers LE, Hoekstra MO, Timmerman HM, van Uden NO, de Graaf PM, Smits HH, Kimpen JL, Rijkers GT. Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. Clin Exp Immunol. 2007. 149:344-52.

Noon, L. Prophylactic inoculation against hay fever. Lancet. 1911. 1572-1573.

Norton PM, Brown HW, Wells JM, Macpherson AM, Wilson PW, Le Page RW. Factors affecting the immunogenicity of tetanus toxin fragment C expressed in Lactococcus lactis. FEMS Immunol Med Microbiol. 1996. 14:167-77.

O'May GA, Macfarlane GT. Probiotic efficacy: are the claims justified? In Probiotic Dairy Products. 2005. 138–166.

O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity. 1998. 8:275-83.

Orla-Jensen S. In: Orla-Jensen S. The lactic acid bacteria. Host, Copenhagen. 1919. 1-196.

O'sullivan DJ, Klaenhammer TR. Rapid Mini-Prep Isolation of High-Quality Plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl Environ Microbiol. 1993. 59:2730-2733.

Pagno GB, Barberio G, de Luca F, Morabito L, Parmiani S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. Clin Exp Allergy 2001. 31:1392–7.

Pajno GB, Caminiti L, Vita D, Barberio G, Salzano G, Lombardo F, Canonica GW, Passalacqua G. Sublingual immunotherapy in mite-sensitized children with atopic dermatitis: a randomized, double-blind, placebo-controlled study. J Allergy Clin Immunol. 2007. 120:164-70.

Pant N, Hultberg A, Zhao Y, Svensson L, Pan-Hammarstrom Q, Johansen K, Pouwels PH, Ruggeri FM, Hermans P, Frenken L, Boren T, Marcotte H, Hammarstrom L. Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. J Infect Dis. 2006. 194:1580-8.

Parker RB. Probiotics, the other half of the antibiotic story. Anim Nutr Health. 1974. 29:4-8.

Passalacqua G, Musarra A, Pecora S, Amoroso S, Antonicelli L, Cadario G, Di Gioacchino M, Lombardi C, Ridolo E, Sacerdoti G, Schiavino D, Senna G. Quantitative assessment of the compliance with once-daily sublingual immunotherapy in children (EASY project: evaluation of a novel SLIT formulation during a year). Pediatr Allergy Immunol. 2007.18:58-62.

Passalacqua G, Pasquali M, Ariano R, Lombardi C, Giardini A, Baiardini I, Majani G, Falagiani P, Bruno M, Canonica GW. Randomized double-blind controlled study with sublingual carbamylated allergoid immunotherapy in mild rhinitis due to mites. Allergy. 2006. 61:849-54.

Pavan S, Hols P, Delcour J, Geoffroy MC, Grangette C, Kleerebezem M, Mercenier A. Adaptation of the nisin-controlled expression system in Lactobacillus plantarum: a tool to study in vivo biological effects. Appl Environ Microbiol. 2000. 66:4427-32.

Peña JA, Versalovic J. Lactobacillus rhamnosus GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. Cell Microbiol. 2003. 5:277-85.

Penders J, Stobberingh EE, Thijs C, Adams H, Vink C, van Ree R, van den Brandt PA. Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was not developing. Clin Exp Allergy. 2006. 36:1602-8.

Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, Adams H, van Ree R, Stobberingh EE. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut. 2007. 56:661-7.

Peng GC, Hsu CH. The efficacy and safety of heat-killed Lactobacillus paracasei for treatment of perennial allergic rhinitis induced by house-dust mite. Pediatr Allergy Immunol. 2005. 16:433-8.

Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity. 1997. 6:411-7.

Plant LJ, Conway PL. Adjuvant properties and colonization potential of adhering and non-adhering Lactobacillus spp following oral administration to mice. FEMS Immunol Med Microbiol. 2002. 34:105-11.

Platts-Mills TA. Mechanisms of bronchial reactivity: the role of immunoglobulin E. Am Rev Respir Dis. 1992. 145:S44-7.

Platts-Mills, de Weck. Dust mite allergens and asthma - a worldwide problem. J. Allergy Clin Immunol. 1989. 100, S2-24.

Pochard P, Hammad H, Ratajczak C, Charbonnier-Hatzfeld AS, Just N, Tonnel AB, Pestel J. Direct regulatory immune activity of lactic acid bacteria on Der p 1-pulsed dendritic cells from allergic patients. J Allergy Clin Immunol. 2005. 116:198-204.

Pohjavuori E, Viljanen M, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, Savilahti E. Lactobacillus GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol. 2004. 114:131-6.

Pouwels PH, Leer RJ, Boersma WJ. The potential of Lactobacillus as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigens. J Biotechnol. 1996. 44:183-92.

Pouwels PH, Leer RJ, Shaw M, Heijne den Bak-Glashouwer MJ, Tielen FD, Smit E, Martinez B, Jore J, Conway PL. Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. Int J Food Microbiol. 1998. 41:155-67.

Pouwels PH, Vriesema A, Martinez B, Tielen FJ, Seegers JF, Leer RJ, Jore J, Smit E. Lactobacilli as vehicles for targeting antigens to mucosal tissues by surface exposition of foreign antigens. Methods Enzymol. 2001. 336:369-89.

Puccio FA, Lynch NR, Noya O, Noda A, Hagel I, López E, López R, Caraballo L, Mercado D, DiPrisco MC. Importance of including Blomia tropicalis in the routine diagnosis of Venezuelan patients with persistent allergic symptoms. Allergy. 2004. 59:753-7.

Puerta Llerena L, Fernández-Caldas E, Caraballo Gracia LR, Lockey RF. Sensitization to Blomia tropicalis and Lepidoglyphus destructor in Dermatophagoides spp-allergic individuals. J Allergy Clin Immunol. 1991. 88:943-50.

Quadri LE. Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria. Antonie Van Leeuwenhoek. 2002. 82:133-45.
Reveneau N, Geoffroy MC, Locht C, Chagnaud P, Mercenier A. Comparison of the immune responses induced by local immunizations with recombinant Lactobacillus plantarum producing tetanus toxin fragment C in different cellular locations. Vaccine. 2002. 20:1769-77.

Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M. The role of interleukin-6 in mucosal IgA antibody responses in vivo. Science. 1994. 264:561-3.

Rautava S, Isolauri E. Cow's milk allergy in infants with atopic eczema is associated with aberrant production of interleukin-4 during oral cow's milk challenge. J Pediatr Gastroenterol Nutr. 2004. 39:529-35.

Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J, Kraft D, Breiteneder H, Mercenier A, Wiedermann U. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. Vaccine. 2003. 22:87-95.

Rienzo VD, Marcucci F, Puccinelli P, Parmiani S, Frati F, Sensi L, Canonica GW, Passalacqua G. Long-lasting effect of sublingual immunotherapy in children with asthma due to house dust mite: a 10-year prospective study. Clin Exp Allergy. 2003. 33:206-10.

Rienzo VD, Minelli M, Musarra A, Sambugaro R, Pecora S, Canonica WG, Passalacqua G. Post-marketing survey on the safety of sublingual immunotherapy in children below the age of 5 years. Clin Exp Allergy. 2005. 35:560-4.

Rigaux P, Daniel C, Hisbergues M, Muraille E, Hols P, Pot B, Pestel J, Jacquet A. Immunomodulatory properties of Lactobacillus plantarum and its use as a recombinant vaccine against mite allergy. Allergy. 2009. 64:406-14.

Rincón M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. J Exp Med. 1997. 185:461-9.

Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, Paerregaard A. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol. 2003. 111:389-95.

Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med. 1995. 182:389-400.

Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994. 179:1109-18.

Sánchez-Borges M, Capriles-Hulett A, Caballero-Fonseca F, Fernández-Caldas E. Mite and cockroach sensitization in allergic patients from Caracas, Venezuela. Ann Allergy Asthma Immunol. 2003. 90:664-8.

Sanders ME. Probiotics: considerations for human health. Nutr Rev. 2003. 61:91-9.

Sanderson CJ. Interleukin-5, eosinophils, and disease. Blood. 1992. 79:3101-9.

Sashihara T, Sueki N, Ikegami S. An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. J Dairy Sci. 2006. 89:2846-55.

Scheppler L, Vogel M, Marti P, Müller L, Miescher SM, Stadler BM. Intranasal immunisation using recombinant Lactobacillus johnsonii as a new strategy to prevent allergic disease. Vaccine. 2005. 23:1126-34.

Scheppler L, Vogel M, Zuercher AW, Zuercher M, Germond JE, Miescher SM, Stadler BM. Recombinant Lactobacillus johnsonii as a mucosal vaccine delivery vehicle. Vaccine. 2002. 20:2913-20.

Schrezenmeir J, de Vrese M. Probiotics, prebiotics, and synbiotics--approaching a definition. Am J Clin Nutr. 2001. 73:361S-364S.

Seegers JF. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol. 2002. 20:508-15.

Sepp E, Julge K, Mikelsaar M, Björkstén B. Intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian children. Clin Exp Allergy. 2005. 35:1141-6.

Sepp E, Julge K, Vasar M, Naaber P, Björksten B, Mikelsaar M. Intestinal microflora of Estonian and Swedish infants. Acta Paediatr. 1997. 86:956-61.

Shaw DM, Gaerthé B, Leer RJ, Van Der Stap JG, Smittenaar C, Heijne Den Bak-Glashouwer M, Thole JE, Tielen FJ, Pouwels PH, Havenith CE. Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli. Immunology. 2000. 100:510-8.

Silva M, Jacobus NV, Deneke C, Gorbach SL. Antimicrobial substance from a human Lactobacillus strain. Antimicrob Agents Chemother. 1987. 31:1231-3.

Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of major house dust mite allergen Der p 2. Mol Immunol. 1996. 33:399-405.

Smith KM, Davidson JM, Garside P. T-cell activation occurs simultaneously in local and peripheral lymphoid tissue following oral administration of a range of doses of immunogenic or tolerogenic antigen although tolerized T cells display a defect in cell division. Immunology. 2002. 106:144-58.

Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, Zaat BA, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol. 2005. 115:1260-7.

Sørvig E, Grönqvist S, Naterstad K, Mathiesen G, Eijsink VG, Axelsson L. Construction of vectors for inducible gene expression in Lactobacillus sakei and L plantarum. FEMS Microbiol Lett. 2003. 229:119-26.

Sørvig E, Mathiesen G, Naterstad K, Eijsink VG, Axelsson L. High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. Microbiology. 2005. 151:2439-49.

Spahn TW, Weiner HL, Rennert PD, Lügering N, Fontana A, Domschke W, Kucharzik T. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. Eur J Immunol. 2002. 32:1109-13.

Stumbles PA, Thomas JA, Pimm CL, Lee PT, Venaille TJ, Proksch S, Holt PG. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. J Exp Med. 1998. 188:2019-31.

Sun BQ, Wu A, Chan A, Chik S, Wong D, Zhong NS. House dust mite allergen (Der p 1 and Blo t 5) levels in asthmatics' home in Hongkong. Chin Med Sci J. 2004. 19:185-8.

Svensson M, Pfeifer J, Stockinger B, Wick MJ. Bacterial antigen delivery systems: phagocytic processing of bacterial antigens for MHC-I and MHC-II presentation to T cells. Behring Inst Mitt. 1997. 197-211.

Takai T, Ichikawa S, Hatanaka H, et al. Effects of proline mutations in the major house dust mite allergen Der f 2 on IgE-binding and histamine-releasing activity. Eur J Biochem. 2000. 267:6650-6656.

Takai T, Yokota T, Yasue M, et al. Engineering of the major house dust mite allergen Der f 2 for allergen specific immunotherapy. Nat Biotechnol. 1997. 15:754-758.

Tao Y, Drabik KA, Waypa TS, Musch MW, Alverdy JC, Schneewind O, Chang EB, Petrof EO. Soluble factors from Lactobacillus GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. Am J Physiol Cell Physiol. 2006. 290:C1018-30. Erratum in: Am J Physiol Cell Physiol. 2006. 291:C194.

Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol. 2007. 119:184-91.

The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. 1998. 351:1225-32.

Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. Int Arch Allergy Immunol. 2002. 129:1–8.

Tissier H. Traitement des infections intestinales par la methode de la flore bacterienne del'intestin. CR Soc Biol. 1906. 60:359–361.

Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. Nature. 1981. 289:592-3.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood. 1994. 84:4008-27.

Ulrik CS, Backer V. Atopy in Danish children and adolescents: results from a longitudinal population study. Ann Allergy Asthma Immunol. 2000. 85:293-7.

van Bronswijk JE, de Cock AW, Oshima S. [The genus Blomia Oudemans (Acari : Glycyphagidae) I. Description of Blomia tropicalis sp. n. from house dust in tropical and sub-tropical regions.] Acarologia. 1974. 15:477-89.

Van Overtvelt L, Lombardi V, Razafindratsita A, Saint-Lu N, Horiot S, Moussu H, Mascarell L, Moingeon P. IL-10-inducing adjuvants enhance sublingual immunotherapy efficacy in a murine asthma model. Int Arch Allergy Immunol. 2008. 145:152-62.

Vidard L, Kovacsovics-Bankowski M, Kraeft SK, Chen LB, Benacerraf B, Rock KL. Analysis of MHC class II presentation of particulate antigens of B lymphocytes. J Immunol. 1996. 156:2809-18.

Viljanen M, Pohjavuori E, Haahtela T, Korpela R, Kuitunen M, Sarnesto A, Vaarala O, Savilahti E. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. J Allergy Clin Immunol. 2005. 115:1254-9.

Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy. 2005. 60:494-500.

von Pirquet C. Allergie. Munchen Med Wehnschr. 1906. 53:1457.

Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity. 2003. 18:605-17.

Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, Umetsu DT. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. J Immunol. 2001. 167:4668-75.

Wang MF, Lin HC, Wang YY, Hsu CH. Treatment of perennial allergic rhinitis with lactic acid bacteria. Pediatr Allergy Immunol 2004. 15:152-158

Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y, Kumemura M. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. J Allergy Clin Immunol. 2003. 111:587-91.

Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev. 2001. 182:207-14.

Weiss KB, Sullivan SD, Lyttle CS. Trends in the cost of illness for asthma in the United States, 1985-1994. J Allergy Clin Immunol. 2000. 106:493-9.

Wells JM, Mercenier A. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol. 2008. 6:349-62.

Werfel T, Breuer K, Ruéff F, Przybilla B, Worm M, Grewe M, Ruzicka T, Brehler R, Wolf H, Schnitker J, Kapp A. Usefulness of specific immunotherapy in patients with atopic dermatitis and allergic sensitization to house dust mites: a multi-centre, randomized, dose-response study. Allergy. 2006. 61:202-5.

Wheeler JG, Shema SJ, Bogle ML, Shirrell MA, Burks AW, Pittler A, Helm RM. Immune and clinical impact of Lactobacillus acidophilus on asthma. Ann Allergy Asthma Immunol. 1997. 79:229-33.

Wills-Karp M, Ewart SL. Time to draw breath: asthma-susceptibility genes are identified. Nat Rev Genet. 2004. 5:376-87.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science. 1998. 282:2258-61.

Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev. 2004. 202:175-90.

Wynn TA. IL-13 effector functions. Annu Rev Immunol. 2003. 21:425-56.

Yang X, Gartner J, Zhu LH, Wang SH, Brunham RC. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection. J. Immunol. 1999. 162:1010–17.

Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y. Cloning and expression of cDNA encoding the complete prepro-form of an isoform of Der f 1, the major group 1 allergen from house dust mite Dermatophagoides farinae. Biosci Biotechnol Biochem. 2001. 65:563-9.

Yeoh SM, Kuo IC, Wang DY, Liam CK, Sam CK, De Bruyne JA, Lee BW, Cheong N, Chua KY. Sensitization profiles of Malaysian and Singaporean subjects to allergens from Dermatophagoides pteronyssinus and Blomia tropicalis. Int Arch Allergy Immunol. 2003. 132:215-20.

Yi FC, Chua KY, Cheong N, Shek LP, Lee BW. Immunoglobulin E reactivity of native Blo t 5, a major allergen of Blomia tropicalis. Clin Exp Allergy. 2004. 34:1762-7.

Yi FC, Lee BW, Cheong N, Chua KY. Quantification of Blo t 5 in mite and dust extracts by two-site ELISA. Allergy. 2005. 60:108-12.

Yi FC, Shek LP, Cheong N, Chua KY, Lee BW. Molecular cloning of Blomia tropicalis allergens--a major source of dust mite allergens in the tropics and subtropics. Inflamm Allergy Drug Targets. 2006. 5:261-6.

Yu MK, Lin CY, Chen WL, Chen CT. Prevalence of Blomia tropicalis in wheezing children in central Taiwan. J Microbiol Immunol Infect. 2008. 41:68-73.

Zegers ND, Kluter E, van Der Stap H, van Dura E, van Dalen P, Shaw M, Baillie L. Expression of the protective antigen of Bacillus anthracis by Lactobacillus casei: towards the development of an oral vaccine against anthrax. J Appl Microbiol. 1999. 87:309-14.

Zeller JC, Panoskaltsis-Mortari A, Murphy WJ, Ruscetti FW, Narula S, Roncarolo MG, Blazar BR. Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. J Immunol. 1999. 163:3684-91.

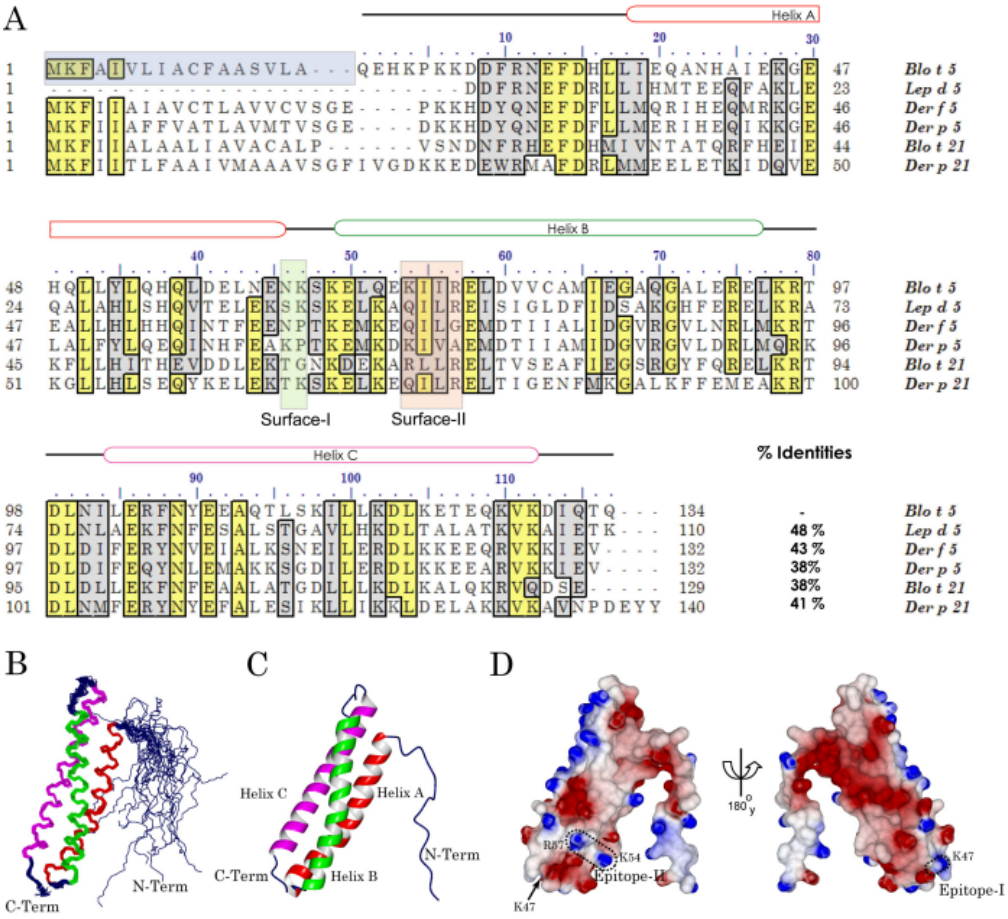
Zhang L, Chew FT, Soh SY, Yi FC, Law SY, Goh DY, Lee BW. Prevalence and distribution of indoor allergens in Singapore. Clin Exp Allergy. 1997. 27:876-85.
Zhou XX, Li WF, Ma GX, Pan YJ.The nisin-controlled gene expression system: construction, application and improvements. Biotechnol Adv. 2006. 24:285-95.

Appendix 1: The cDNA sequence of *Blomia tropicalis* group 5 allergen

```
1   atgaagttcg ccatcgttct tattgcctgc tttgccgctt cggttttggc tcaagagcac
61  aagccaaaga aggatgattt ccgaaacgaa ttcgatcact tgttgatcga acaggcaaac
121 catgctatcg aaaagggaga acatcaattg ctttacttgc aacaccaact cgacgaattg
181 aatgaaaaca agagcaagga attgcaagag aaaatcattc gagaacttga tgttgtttgc
241 gccatgatcg aaggagccca aggagctttg gaacgtgaat tgaagcgaac tgatcttaac
301 attttggaac gattcaacta cgaagaggct caaactctca gcaagatctt gcttaaggat
361 ttgaaggaaa ccgaacaaaa agtgaaggat attcaaaccc aataa
```

Appendix 1. The cDNA sequence of *Blomia tropicalis* group 5 allergen (Blo t 5). This nucleotide sequence is from the GenBank with accession no: U59102. The cDNA sequence of Blo t 5 contains an open reading frame of 405 base pairs that encoded for the Blo t 5 signal peptide (17 residues; 1-51) and the mature Blo t 5 protein (117 residues; 52-405).

Appendix 2: The NMR Solution Structure of Blo t 5.



Appendix 2. The NMR Solution Structure of Blo t 5. (A) The primary sequence alignment of the Group 5 and the Group 21 allergens from the different mite species, viz. *Blomia tropicalis*, *Lepidoglyphus destructor*, *Dermatophagoides farina*, and *D. pteronyssinus*. The sequence identities and the conservative substitutions are shaded yellow and gray, respectively, and the percentage identity with respect to Blo t 5 is shown at the end. The numbers on the left and the right sides of the sequences are indicative of the residue numbering for the proteins encoded in the cDNA sequence deposited in the databases with the following accession numbers: Blo t 5, O96870; Der f 5, BAE45865; Der p 5, P14004; Lep d 5, Q9U5P2; Der p 21, ABC73706; and Blo t 21, AY800348. The 17 residue leader peptide of Blo t 5 is boxed, and the residues of mature Blo t 5 are numbered in blue on the top along with the secondary structure. The critical residues of the mAb 4A7 epitope are located on surfaces I and II as indicated. (B) The NMR solution structure of Blo t 5 shown as an ensemble of 20 conformers overlaid with the backbone atoms of the helical residues Leu18–Asp113 on the lowest-energy first conformer. (C) The ribbon representation of the lowest-energy conformer of

Blo t 5. The three helices are numbered A, B, and C and are colored red, green, and purple, respectively. (D) Two orientations of the Blo t 5 surface charge distribution generated by a 180º flip along the y axis and colored with blue for positive charge and red for negative charge.

(Adapted from Naik MT, Chang CF, Kuo IC, Kung CC, Yi FC, Chua KY, Huang TH. Roles of structure and structural dynamics in the antibody recognition of the allergen proteins: an NMR study on *Blomia tropicalis* major allergen. Structure 2008 16(1):125-36.)

Appendix 3: Culture media used for *Lactococcus lactis* MG 1363

Sterilize all media by autoclaving.

M17 broth
0.5% tryptone
0.5% soya peptone
0.5% meat digest
0.25% yeast extract
0.05% ascorbic acid
0.025% magnesium sulfate
1.9% disodium glycerophosphate

GM17 broth
M17 medium
0.5% glucose

SGM17 broth
GM17 medium
0.5 M sucrose

SGM17MC
SGM17 medium
20 mM $MgCl_2$
2 mM $CaCl_2$

GM17 agar
GM17 medium
1.5% agar

SR plate
1% tryptone
0.5% yeast extract
20% sucrose
1% glucose
2.5% gelatin
1.5% agar
2.5 mM $MgCl_2$
2.5 mM $CaCl_2$

Appendix 4: Culture media used for *Lactobacillus* strains

Sterilize all media by autoclaving.

MRS broth

1 % peptone
0.8 % meat extract
0.4 % yeast extract
2% glucose
1 mL of monooleate (Tween 80)
0.2% potassium phosphate, dibasic
0.5 % sodium acetate, trihydrate
0.2 % ammonium citrate, tribasic
0.02 % magnesium sulphate, heptahydrate
0.005% manganese sulphate, tetrahydrate

MRSSM broth

MRS medium
0.5 M sucrose
0.1 mM $MgCl_2$

MRS agar

MRS medium
1.5% agar

Appendix 5: DNA sequence of the pSIP412 expression vector

```
   1 GAATTCGGTA CCCCGGGTTC GAAGGCGCCA AGCTTCAAAT TACAGCACGT GTTGCTTTGA
  61 TTGATAGCCA AAAAGCAGCA GTTGATAAAG CAATTACTGA TATTGCTGAA AAATTGTAAT
 121 TTATAAATAA AAATCACCTT TTAGAGGTGG TTTTTTTATT TATAAATTAT TCGTTTGATT
 181 TCGCTTTCGA TAGAACAATC AAAGCGAGAA TAAGGAAGAT AAATCCCATA AGGGCGGGAG
 241 CAGAATGTCC GAGACTAATT CATGAGATCG ATTTTTTATT AAAACGTCTC AAAATCGTTT
 301 CTGAGACGTT TTAGCGTTTA TTTCGTTTAG TTATCGGCAT AATCGTTAAA ACAGGCGTTA
 361 TCGTAGCGTA AAAGCCCTTG AGCGTAGCGT GCTTTGCAGC GAAGATGTTG TCTGTTAGAT
 421 TATGAAAGCC GATGACTGAA TGAAATAATA AGCGCAGCGT CCTTCTATTT CGGTTGGAGG
 481 AGGCTCAAGG GAGTTTGAGG GAATGAAATT CCCTCATGGG TTTGATTTTA AAAATTGCTT
 541 GCAATTTTGC CGAGCGGTAG CGCTGGAAAA ATTTTTGAAA AAAATTTGGA ATTTGGAAAA
 601 AAATGGGGGG AAAGGAAGCG AATTTTGCTT CCGTACTACG ACCCCCCATT AAGTGCCGAG
 661 TGCCAATTTT TGTGCCAAAA ACGCTCTATC CCAACTGGCT CAAGGGTTTG AGGGGTTTTT
 721 CAATCGCCAA CGAATCGCCA ACGTTTTCGC CAACGTTTTT TATAAATCTA TATTTAAGTA
 781 GCTTTATTGT TGTTTTTATG ATTACAAAGT GATACACTAA TTTTATAAAA TTATTTGATT
 841 GGAGTTTTTT AAATGGTGAT TTCAGAATCG AAAAAAAGAG TTATGATTTC TCTGACAAAA
 901 GAGCAAGATA AAAAATTAAC AGATATGGCG AAACAAAAAG GTTTTTCAAA ATCTGCGGTT
 961 GCGGCGTTAG CTATAGAAGA ATATGCAAGA AAGGAATCAG AATAAAAAAA ATAAGCGAAA
1021 GCTCGCGTTT TTAGAAGGAT ACGAGTTTTC GCTACTTGTT TTTGATAAGG TAATATATCA
1081 TGGCTATTAA ATACTAAAGC TAGAAATTTT GGATTTTTAT TATATCCTGA CTCAATTCCT
1141 AATGATTGGA AAGAAAAATT AGAGAGTTTG GGCGTATCTA TGGCTGTCAG TCCTTTACAC
1201 GATATGGACG AAAAAAAAGA TAAAGATACA TGGAATAGTA GTGATGTTAT ACGAAATGGA
1261 AAGCACTATA AAAAACCACA CTATCACGTT ATATATATTG CACGAAATCC TGTAACAATA
1321 GAAAGCGTTA GGAACAAGAT TAAGCGAAAA TTGGGGAATA GTTCAGTTGC TCATGTTGAG
1381 ATACTTGATT ATATCAAAGG TTCATATGAA TATTTGACTC ATGAATCAAA GGACGCTATT
1441 GCTAAGAATA AACATATATA CGACAAAAAA GATATTTTGA ACATTAATGA TTTTGATATT
1501 GACCGCTATA TAACACTTGA TGAAAGCCAA AAAAGAGAAT TGAAGAATTT ACTTTTAGAT
1561 ATAGTGGATG ACTATAATTT GGTAAATACA AAAGATTTAA TGGCTTTTAT TCGCCTTAGG
1621 GGAGCGGAGT TTGGAATTTT AAATACGAAT GATGTAAAAG ATATTGTTTC AACAAACTCT
1681 AGCGCCTTTA GATTATGGTT TGAGGGCAAT TATCAGTGTG GATATAGAGC AAGTTATGCA
1741 AAGGTTCTTG ATGCTGAAAC GGGGGAAATA AAATGACAAA CAAAGAAAAA GAGTTATTTG
1801 CTGAAAATGA GGAATTAAAA AAAGAAATTA AGGACTTAAA AGAGCGTATT GAAAGATACA
1861 GAGAAATGGA AGTTGAATTA AGTACAACAA TAGATTTATT GAGAGGAGGG ATTATTGAAT
1921 AAATAAAAGC CCCCCTGACG AAAGTCGAAG GGGGCTTTTA TTTTGGTTTG ATGTTGCGAT
1981 TAATAGCAAT ACGATTGCAA TAAACAAAAT GATCCCCTTA GAAGCAAACT TAAGAGTGTG
2041 TTGATAGTGC ATTATCTTAA AATTTTGTAT AATAGGAATT GAAGTTAAAT TAGATGCTAA
2101 AAATAGGAAT TGAAGTTAAA TTAGATGCTA AAAATTTGTA ATTAAGAAGG AGGGATTCGT
2161 CATGTTGGTA TTCCAAATGC GTAATGTAGA TAAAACATCT ACTGTTTTGA AACAGACTAA
2221 AAACAGTGAT TACGCAGATA AATAAATACG TTAGATTAAT TCCTACCAGT GACTAATCTT
2281 ATGACTTTTT AAACAGATAA CTAAAATTAC AAACAAATCG TTTAACTTCA GGAGAGATTA
2341 CATGAACAAA AATATAAATA TCTCAAACTT TTTAACGAGT GAAAAAGTAC TCAACCAAAT
2401 AATAAAACAA TTGAATTTAA AAGAAACCGA TACCGTTTAC GAAATTGGAA CAGGTAAAGG
2461 GCATTTAACG ACGAAACTGG CTAAAATAAG TAAACAGGTA ACGTCTATTG AATTAGACAG
```

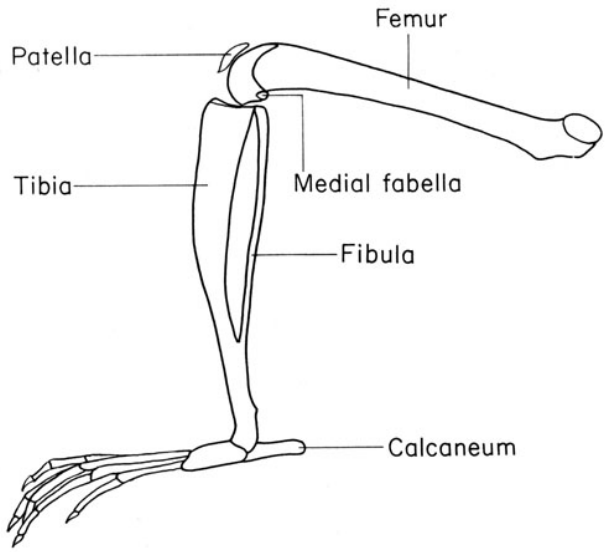
2521 TCATCTATTC AACTTATCGT CAGAAAAATT AAAACTGAAT ACTCGTGTCA CTTTAATTCA
2581 CCAAGATATT CTACAGTTTC AATTCCCTAA CAAACAGAGG TATAAAATTG TTGGGAATAT
2641 TCCTTACAAT TTAAGCACAC AAATTATTAA AAAAGTGGTT TTTGAAAGCC GTGCGTCTGA
2701 CATCTATCTG ACTGTTGAAG AAGGATTCTA CAAGCGTACC TTGGATATTC ACCGAACACT
2761 AGGGTTGCTC TTGCACACTC AAGTCTCGAT TCAGCAATTG CTTAAGCTGC CAGCGGAATG
2821 CTTTCATCCT AAACCAAAAG TAAACAGTGT CTTAATAAAA CTTACCCGCC ATACCACAGA
2881 TGTTCCAGAT AAATATTGGA AGCTATATAA GTACTTTGTT TCAAAATGGG TCAATCGAGA
2941 ATATCGTCAA CTGTTTACTA AAAATCAGTT TCGTCAAGCA ATGAAACACG CCAAAGTAAA
3001 CAATTTAAGT ACCATTACTT ATGAGCAAGT ATTGTCTATT TTTAATAGTT ATCTATTATT
3061 TAACGGGAGG AAATAATTCT ATGAGTCGCT TTTTTAAATT TGGAAAGTTA CACGTTACTA
3121 AAGGGAATGG AGACCGGGGT CGACCCTTCA ATAGAGTTCT TAACGTTAAT CCGAAAAAAA
3181 CTAACGTTAA TATTAAAAAA TAAGATCCGC TTGTGAATTA TGTATAATTT GATTAGACTA
3241 AAGAATAGGA GAAAGTATGA TGATATTTAA AAAACTTTCT CGTTAAGATA GGTTGTTGGT
3301 GAGCATGTTA TATACGGATG TATCGGTTTC CTTAATGCAA AATTTTGTTG CTATCTTATT
3361 AATTTTTCTA TTATATAGAT ATATTCAAAG AAAGATAACA TTTAAACGGA TCATATTAGA
3421 TATTTTAATA GCGATTATTT TTTCAATATT ATATCTGTTT ATTTCAGATG CGTCATTACT
3481 TGTAATGGTA TTAATGCGAT TAGGGTGGCA TTTTCATCAA CAAAAAGAAA ATAAGATAAA
3541 AACGACTGAT ACAGCTAATT TAATTCTAAT TATCGTGATC CAGTTATTGT TAGTTGCGGT
3601 TGGGACTATT ATTAGTCAGT TTACCATATC GATTATCAAA AGTGATTTCA GCCAAAATAT
3661 ATTGAACAAT AGTGCAACAG ATATAACTTT ATTAGGTATT TTCTTTGCTG TTTTATTTGA
3721 CGGCTTGTTC TTTATATTAT TGAAGAATAA GCGGACTGAA TTACAACATT TAAATCAAGA
3781 AATCATTGAA TTTTCGTTAG AAAAACAATA TTTTATATTT ATATTTATTT TATTTATAGT
3841 AATAGAAATT ATTTTAGCAG TTGGGAATCT TCAAGGAGTA ACAGCCACGA TATTATTAAC
3901 CATTATCATT ATTTTTTGTG TCCTTATCGG GATGACTTTT TGGCAAGTGA TGCTTTTTTT
3961 GAAGGCTTAT TCGATTCGCC AAGAAGCCAA TGACCAATTG GTCCGGAATC AACAACTTCA
4021 AGATTATCTA GTCAATATCG AACAGCAGTA CACCGAATTA CGGCGATTTA AGCATGATTA
4081 TCAAAACATC TTATTATCGT TGGAGAGTTT TGCCGAAAAG GGCGATCAGC AACAGTTTAA
4141 GGCGTATTAC CAAGAATTAT TAGCACAACG GCCAATTCAA AGTGAAATCC AAGGGGCAGT
4201 CATTGCACAA CTCGACTACT TGAAAAATGA TCCTATTCGA GGATTAGTCA TTCAAAAGTT
4261 TTTGGCAGCC AAACAGGCTG GTGTTACTTT AAAATTCGAA ATGACCGAAC CAATCGAATT
4321 AGCAACCGCT AATCTATTAA CGGTTATTCG GATTATCGGT ATTTTATTAG ACAATGCGAT
4381 TGAACAAGCC GTTCAAGAAA CCGATCAATT GGTGAGTTGT GCTTTCTTAC AATCTGATGG
4441 TTTAATCGAA ATTACGATTG AAAATACGGC CAGTCAAGTT AAGAATCTCC AAGCATTTTC
4501 AGAGTTAGGC TATTCAACGA AAGGCGCTGG TCGGGGGACT GGTTTAGCTA ATGTGCAGGA
4561 TTTGATTGCC AAACAAACCA ATTTATTCTT AGAAACACAG ATTGAAAATA GAAAGTTACG
4621 ACAGACATTG ATGATTACGG AGGAAACTTA ATTTGTATCC CGTTTATTTA TTAGAGGATG
4681 ATTTACAGCA ACAAGCGATT TATCAGCAAA TTATCGCGAA TACGATTATG ATTAACGAAT
4741 TTGCAATGAC TTTAACATGC GCTGCCAGTG ATACTGAGAC ATTGTTGGCG GCAATTAAGG
4801 ATCAGCAACG AGGTTTATTC TTTTTGGATA TGGAAATTGA GGATAACCGC CAAGCCGGTT
4861 TAGAAGTGGC AACTAAGATT CGGCAGATGA TGCCGTTTGC GCAAATTGTC TTCATTACAA
4921 CCCACGAGGA ACTGACATTA TTAACGTTAG AACGAAAAAT AGCGCCTTTA GATTACATTC
4981 TCAAGGACCA AACAATGGCT GAAATCAAAA GGCAATTGAT TGATGATCTA TTGTTAGCTG
5041 AGAAGCAAAA CGAGGCGGCA GCGTATCACC GAGAAAATTT ATTTAGTTAT AAAATAGGTC
5101 CTCGCTTTTT CTCATTACCA TTAAAGGAAG TTGTTTATTT ATATACTGAA AAAGAAAATC

5161 CGGGTCATAT TAATTTGTTA GCCGTTACCA GAAAGGTTAC TTTTCCAGGA AATTTAAATG
5221 CGCTGGAAGC CCAATATCCA ATGCTCTTTC GGTGTGATAA AAGTTACTTA GTTAACCTAT
5281 CTAATATTGC CAATTATGAC AGTAAAACAC GGAGTTTAAA ATTTGTAGAT GGCAGTGAGG
5341 CAAAAGTCTC GTTCCGGAAA TCACGGGAAC TAGTGGCCAA ATTAAAACAA ATGATGTAGC
5401 GCCTGCAGGC ACGCCAAATG ATCCCAGTAA AAAGCCACCC GCATGGCGGG TGGCTTTTTA
5461 TTAGCCCTAG AAGGGCTTCC CACACGCATT TCAGCGCCTT AGTGCCTTAG TTTGTGAATC
5521 ATAGGTGGTA TAGTCCCGAA ATACCCGTCT AAGGAATTGT CAGATAGGCC TAATGACTGG
5581 CTTTTATAAT ATGAGATAAT GCCGACTGTA CTTTTTACAG TCGGTTTTCT AATGTCACTA
5641 ACCTGCCCCG TTAGTTGAAG AAGGTTTTTA TATTACAGCT CCAGATCTAC CGGTTTAATT
5701 TGAAAATTGA TATTAGCGTT TAACAGTTAA ATTAATACGT TAATAATTTT TTTGTCTTTA
5761 AATAGGGATT TGAAGCATAA TGGTGTTATA GCGTACTTAG CTGGCCAGCA TATATGTATT
5821 CTATAAAATA CTATTACAAG GAGATTTTAG **CCATGG**CTGT AAAACGTTTA ATTGAAACTT
5881 TTGTTCCGGA AAATTATAAA ATTTTCCTTG ATATTGACCG TAAAACTAAG AAAATAAAAG
5941 GTCAAGTGGC AATTACTGGG GAAGCAAAAG ATACTGTTGT TGCCTTTCAT GCCAAAGGAT
6001 TACACTTCAA CAAAGTTCGC GCTTTTAGTG TTGATACAAA CTTCATTGAA AATGAAGAAG
6061 ATGAAGAAAT TGTTGTTAAA ATTGGTGAAA CAGGGCGCGT GACTGTTTCA TTTGAATATG
6121 AAGCCGAATT GACTGACAAC ATGATGGGAA TCTATCCTTC ATATTATGAA GTCAATGGTG
6181 AAAAGAAAAT GCTAATTGGC ACACAGTTTG AAAGCCATTT TGCTCGCCAA GCTTTCCCAT
6241 CTATTGATGA ACCAGAAGCG AAAGCAACTT TCGATTTGTC AGTAAAATTT GATGAAGAAG
6301 AAGGCGACAT CATTGTTTCA AATATGCCAG AACTCTTGAA TATTAATGGA ATTCACGTTT
6361 TTGAACGGAC AGTTAAAATG AGTTCTTACC TTTTGGCTTT TGTATTCGGT GAACTTCAAT
6421 ATAAAAAAGG AAAAACAAAA TCTGGTGTTG AAGTAGGTGC CTTTGCAACG AAAGCTCATA
6481 GTCAAGCAGC ACTTGATTTC CCACTTGATA TTGCTATTCG TTCAATCGAA TTTTACGAAG
6541 ATTATTATCA AACACCCTAT CCACTTCCAC ACAGCTGGCA TATTGCCTTG CCTGACTTCT
6601 CATCAGGAGC AATGGAAAAC TGGGGATGTA TCACTTATCG TGAAGTTTGC ATGTTGGTTG
6661 ACCCTGAAAA TGCAACCATT CAAAGTAAAC AATATGTGGC AACAGTTATT GCCCATGAAT
6721 TGGCTCACCA GTGGTTCGGT GACCTTGTAA CTATGCAATG GTGGGATGAT TTGTGGCTCA
6781 ACGAATCATT CGCTAACAAC ATGGAATATG TTTGTATGGA TGCTTTGGAA CCAAGTTGGA
6841 ACGTTTGGGA ATCATTCTCA ATTTCAGAAG CCAATATGGC ATTGAATCGT GATGCAACTG
6901 ATGGAGTTCA ATCTGTCCAC GTTGAAGTAA CTCACCCAGA TGAAATTGGA ACACTTTTTG
6961 ATCCAGCAAT CGTCTATGCT AAAGGTTCAC GCTTAATGGT TATGCTTCGT AAATGGCTCG
7021 GAGATGAAGA CTTTGCTGCT GGCTTAGCCC TTTATTTCAA ACGCCATCAA TATGGAAATA
7081 CAGTTGGAGA CAATCTTTGG GATGCCTTGG CTGAAGTTTC TGGAAAAGAT GTGGCAGCCT
7141 TCATGCACTC ATGGGTTAAT CAACCGGGTT ACCCTGTCGT TACAGCAGAA GTCGTTGATG
7201 ATACTTTGAT TTTGAGCCAA AAACAATTCT TTGTTGGTGA AGGTGTTGAC AAAGGACGTT
7261 TGTGGAATGT TCCATTGAAT ACCAATTGGA CAGGGCTACC AGACTTGCTT TCTTCTGAAA
7321 AAGTTGAAAT TCCAGGGTTT GCTGCTTTGA AAACTAAAAA CAATGGTAAA GCTCTCTTTT
7381 TGAATGATGC AAATATGGCT CATTACATCA TTGATTATAA GGGTGCTTTA CTGACAGACC
7441 TTCTTTCAGA AGTTGAATCT TTGGAGAATG TGACAAAATT CCAAATCTTG CAAGACCGTA
7501 AATTGTTGGC TAAAGCAGGT GTGATTTCTT ATGCTGACGT TGTAAATATC TTGCCATCAT
7561 TCACTAATGA AGAATCTTAC CTTGTTAATA CTGGTTTGAG TCAACTCATT AGTGAGTTGG
7621 AACTTTTTGT TGATGAAGAT TCAGAAACTG AAAAAGCCTT CCAAAGCTTG GTAGGAAAAC
7681 TTTTTGCTAA AAATTATGCT CGTTTGGGTT GGGATAAAGT TGCTGGTGAA TCTGCTGGGG
7741 ATGAGAGCCT TCGTGGAATC GTGTTGAGTA AAACTTTGTA TTCTGAAAAT GCAGATGCCA

7801 AAACAAAAGC TAGCCAAATT TTTGCAACAC ATAAAGAAAA TTTAGCAAGT ATTCCAGCTG
7861 ATATTCGTCC AATTGTTTTG AACAATGAAA TCAAAACAAC TAACTCGGCT GAATTGGTTA
7921 AAACTTATCG TGAAACTTAT ATCAAAACAA GCCTCCAAGA ATTCAAACGT GAACTTGAAG
7981 GGGCAGTTGC TTTGATTAAA GATGAAAAAG TTATTGCTGA ATTACTTGAA AGCTTCAAAA
8041 ATGCCGATAT TGTTAAACCA CAAGATATTG CTTTCTCTTG GTTCTATCTT TTGCGCAATG
8101 ATTTTTCACA AGATGCGGCA TGGGCTTGGG AAAAAGCAAA TTGGGCTTTC CTTGAAGAAA
8161 AATTGGGTGG TGATATGAGT TATGACAAAT TTGTCATCTA TCCAGGGAAT ACTTTCAAAA
8221 CTGCTGATAA ATTAGCGGAA TATAAAGCTT TCTTTGAACC AAAATTAGAA AACCAAGGCT
8281 TGAAACGTTC AATTGAAATG GCAATTAAAC AAATTACAGC ACGTGTTGCT TTGATTGATA
8341 GCCAAAAAGC AGCAGTTGAT AAAGCAATTA CTGATATTGC TGAAAAATTG TAATCTAGA**C**
8401 **TCGAG**

Appendix 5. DNA sequence of the pSIP412 expression vector (8405 bp). Sequence coding for MCS: 1-30; *pepN* terminator: 40-260; pSH71 replicon: 260-2010; ermB: 2010-3140; sppKR regulatory genes, 3140-5410; double terminator, 5410-5690; P_{orfx} promoter: 5690-5852; pepN reporter gene (2553 bp): 5853-8405. *NcoI* (CCATGG; 5851-5856) and *XhoI* (CTCGAG; 8399-8405) restriction sites are bold and underlined.

Appendix 6: Skeletons of mouse right hind limb



Appendix 6. Skeletons of mouse right hind limb. The bone marrow cells were flushed out from the femora and tibiae of the hind limbs as the source of cells to derive dendritic cells *in vitro*. (Adapted from Margaret J. Cook, The Anatomy of the Laboratory Mouse, 1965).